#8

PATENT
Docket No.: **21829/230 (EBC-015)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Zhong-Min Wei	Examiner: A. Kubelik
Serial No.	:	10/034,158	
Cnfrm. No.	:	9509	Art Unit: 1638
Filed	:	December 20, 2001	
For	:	METHOD OF IMPARTING DROUGHT RESISTANCE TO PLANTS	

DECLARATION OF ZHONG-MIN WEI UNDER 37 C.F.R. § 1.132

U.S. Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

Dear Sir:

I, ZHONG-MIN WEI, pursuant of 37 C.F.R. § 1.132, declare:

1. I received a B.S. degree in Biology from Zhejiang University, Zhejiang, China in 1982, an M.S. degree in Plant Pathology from Nanjing Agricultural University, Nanjing, China in 1984, and a Ph.D. degree in Molecular Biology from Nanjing Agricultural University and Academy of Science, Shanghai, China in 1987.

2. I am currently employed as Chief Scientific Officer and Vice President of Research and Development at EDEN Bioscience Corporation in Bothell, Washington.

3. I am the inventor of the above-identified application.

4. I am presenting this declaration to show that hypersensitive response elicitors from a diverse range of plant pathogenic bacteria (1) are an art-recognized class of proteins where results achieved with one such protein would be expected when other proteins in this class are used and (2) share the unique ability to cause distinct plant responses. Specifically, treatment of a variety of plants and plant seeds with hypersensitive response elicitors was shown to induce plant disease resistance, enhance plant growth, and induce

plant stress resistance, as compared with plants and plant seeds not treated with a hypersensitive response elicitor.

5. In plants, the hypersensitive response phenomenon results from an incompatible interaction between plant pathogens and non-host plants. As explained in Gopalan et al., "Bacterial Genes Involved in the Elicitation of Hypersensitive Response and Pathogenesis," Plant Disease 80: 604-10 (1996) ("Gopalan") (attached hereto as **Exhibit 1**), these types of interactions involve, for example, a bacterial plant pathogen attempting to infect a host plant, and the host plant preventing proliferation of the pathogen by the collapse and death, or necrosis, of plant leaf cells at the site of infection. This is distinct from a compatible interaction between a bacterial plant pathogen and a host plant in which the bacteria is capable of proliferation, resulting in the spread of the pathogen throughout the plant and the manifestation of disease symptoms. Id. at 604.

6. Hypersensitive response elicitors within a given genus are often homologous to elicitors from different pathogenic species and strains of the same genus. For example, homologs of hypersensitive response elicitors from *Erwinia amylovora* and *Pseudomonas syringae* have been identified in different bacteria species and strains from the genera *Erwinia* and *Pseudomonas*, respectively. See Gopalan.

7. In addition, numerous reported studies confirm that a gene encoding a hypersensitive response elicitor from a particular source genus can be used to isolate a corresponding hypersensitive response elicitor gene from different species and strains of that same genus. For example, in Bauer et al., "*Erwinia chrysanthemi* $\text{Harpin}_{\text{Ech}}$: An Elicitor of the Hypersensitive Response that Contributes to Soft-Rot Pathogenesis," MPMI 8(4): 484-91 (1995) ("Bauer") (attached hereto as **Exhibit 2**), the *Erwinia amylovora* hypersensitive response elicitor encoding gene was used as a probe to isolate, clone, and sequence the gene encoding the *Erwinia chrysanthemi* hypersensitive response elicitor, as follows:

> The cosmids were probed in colony blots with a 1.3-kb *Hind*III fragment from pCPP1084, which contains the *E. amylovora hrpN* gene (Wei et al. [, "Harpin Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (]1992[)]). pCPP2157, one of the three cosmids hybridizing with the probe, was digested with several restriction enzymes, and the location of the $hrpN_{Ech}$ gene in those fragments was determined by probing a Southern blot with *E. amylovora Hind*III fragment. Two fragments, each containing the entire $hrpN_{Ech}$ gene, were subcloned into different vectors: pCPP2142 contained an 8.3-kb *Sal*I fragment in pUC119 (Vieira and Messing [,"Production of Single-Stranded Plasmid DNA," Methods Enzymol., 153:3-11(]

> 1987[)]), and pCPP2141 contained a 3.1-kb *Pst*I fragment in pBluescript II SK(-) (Stratagene, La Jolla, CA).
>
> ***Sequence of hrpN$_{Ech}$***
>
> The nucleotide sequence of a 2.4-kb region of pCPP2141 encompassing *hrpN$_{Ech}$* was determined. The portion of that sequence extending from the putative ribosome-binding site through the *hrpN$_{Ech}$* coding sequence to a putative rho-independent terminator is presented in Figure 1.

See page 485.

8. In the same manner as described in Bauer *supra*, Cui et al., "The RsmA$^-$ Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrp*N$_{Ecc}$ and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI 9(7): 565-73 (1996) ("Cui") (attached hereto as **Exhibit 3**) demonstrates that the gene encoding the *Erwinia carotovora* hypersensitive response elicitor can be isolated, sequenced, and cloned by using the *Erwinia chrysanthemi* hypersensitive response elicitor encoding gene to probe the genomic library of *Erwinia carotovora*. Further, Cui (at page 572) states the following:

> The genomic library of *E. carotovora* subsp. *carotovora* strain Ecc71 in pLARF5 was screened by in situ colony hybridization with a 0.75-kb internal *Cla*I fragment of *hrpN* of *E. chrysanthemi* (Bauer et al.[, "*Erwinia chrysanthemi* Harpin$_{Ech}$: An Elicitor of the Hypersensitive Response that Contributes to Soft-Rot Pathogenesis," MPMI 8(4): 484-91 (]1995). Two cosmids, pAKC921 and pAKC922, that hybridized with the probe were isolated. The subclones (pAKC923 and pAKC924, Table 1) carrying *hrpN* DNA were used for sequence analysis.

9. The gene encoding the hypersensitive response elicitor of *Erwinia amylovora* has also been used as a probe to isolate and clone the gene encoding the hypersensitive response elicitor of *Erwinia stewartii*. It was found that antibodies raised against the hypersensitive response elicitor of *Erwinia stewartii* cross-reacted with the hypersensitive response elicitor of *Erwinia amylovora*. See Ahmad et al., "Harpin Is Not Necessary for the Pathogenicity of Maize," 8th Int'l Cong. Molec. Plant Microbe Inter. July 14-19, 1996 ("Ahmad") (attached hereto as **Exhibit 4**).

10. Similar findings were reported for hypersensitive response elicitors from the genus *Pseudomonas*. An internal fragment of the hypersensitive response elicitor from *Pseudomonas syringae* pv. *syringae* (i.e., *hrpZ*) was used to identify and isolate the hypersensitive response elicitors from *P. syringae* pv. *glycinea* and *P. syringae* pv. *tomato*.

Significant amino acid sequence similarities were identified between the various *Pseudomonas syringae* elicitors. See Preston et al., "The HrpZ Proteins of *Pseudomonas syringae* pvs. *syringae, glycinea,* and *tomato* Are Encoded by an Operon Containing *Yersinia ysc* Homologs and Elicit the Hypersensitive Response in Tomato But Not Soybean," MPMI 8(5): 717-32 (1995) ("Preston") (attached hereto as **Exhibit 5**).

11. The genes encoding hypersensitive response elicitors are positioned within the *hrp* gene cluster or proximate to the *hrp* gene cluster in *hrp* regulons. For example, *hrpN* from *Erwinia amylovora* was located within the *hrp* gene cluster, as was *hrpZ* from *Pseudomonas syringae.* The *popA* gene, encoding a hypersensitive response elicitor from *Pseudomonas solanacearum,* was located on the left flank of the *hrp* gene cluster within a *hrp* regulon. See Bonas, "*hrp* Genes of Phytopathogneic Bacteria," Current Topics in Microbiology and Immunology 192: 79-98 (1994) ("Bonas I") (attached hereto as **Exhibit 6)** and Alfano et al., "The Type III (Hrp) Secretion Pathway of Plant Pathogenic Bacteria: Trafficking Harpins, Avr Proteins, and Death," Journal of Bacteriology 179: 5655-5662 (1997) ("Alfano") (attached hereto as **Exhibit 7**). Similar to the *popA* gene, *hreX,* the gene encoding the hypersensitive response elicitor from *Xanthomonas campestris,* was located on the left flank of the *hrp* gene cluster. See Swanson et al., "Isolation of the *hreX* Gene Encoding the HR Elicitor Harpin (Xcp) from *Xanthomonas campestris* pv. *pelargonii,*" Phytopathology 90: s75 (1999) ("Swanson") (attached hereto as **Exhibit 8**).

12. The characteristics that distinguish hypersensitive response elicitors as a distinct class of molecules are clearly apparent when considering the different elicitors' secretion mechanisms, regulation, biochemical characteristics, and biological activities.

13. Substantially all hypersensitive response elicitors identified have been shown to be secreted through the type III, *hrp* dependent secretion pathway. The type III secretion pathway is a highly conserved and unique mechanism for the delivery of pathogenicity related molecules in gram-negative bacteria. The *hrp* gene cluster is largely composed of components of the type III secretion system. See Bogdanove et al., "Unified Nomenclature for Broadly Conserved *hrp* Genes of Phytopathogenic Bacteria," Molec. Microbiol. 20:681-83 (1996) ("Bogdanove") (attached hereto as **Exhibit 9**); and Alfano.

14. Regulation of the genes encoding the *hrp* gene cluster, and subsequently the genes encoding the components of the type III secretion system and hypersensitive response elicitors, is controlled by environmental factors. Specifically, transcriptional expression of these genes is induced under conditions that mimic the plant apoplast, such as low concentrations of carbon and nitrogen, low temperature, and low pH.

See Wei et al., "Regulation of hrp Genes and Type III Protein Secretion in *Erwinia amylovora* by HrpX/HrpY, a Novel Two-Component System, and HrpS," MPMI 13(11): 1251-1262 (2000) ("Wei I") (attached hereto as **Exhibit 10**); and Bonas I.

15. Biochemically, hypersensitive response elicitors have a number of common characteristics. These include being glycine rich, heat stable, hydrophilic, lacking of an N-terminal signal sequence, and susceptible to proteolysis. See Bonas, "Bacterial Home Goal by Harpins," Trends Microbiol 2: 1-2 (1994) ("Bonas II") (attached hereto as **Exhibit 11**); Bonas I; Gopalan; and Alfano.

16. In addition, hypersensitive response elicitors share a unique secondary structure that has been associated with these elicitors' distinct biological activities (described below). The structure has two primary components, an alpha helix unit and a relaxed acidic unit having a sheet or random turn structure. In the absence of one or both of these components, hypersensitive response elicitation does not occur. See WO 01/98501 to Fan et al. ("Fan") (attached hereto as **Exhibit 12**).

17. In addition to eliciting the hypersensitive response in a broad range of plant species, as explained by Wei et al., "Harpin from *Erwinia amylovora* Induced Plant Resistance," Acta Horticulture 411: 223-225 (1996) ("Wei II") (attached hereto as **Exhibit 13**) and by Alfano, hypersensitive response elicitors also share the ability to induce specific plant responses. The induction of plant disease resistance, plant growth enhancement, and plant stress resistance are three plant responses that result from treatment of plants or plant seeds with a hypersensitive response elicitor from a gram-negative plant pathogen.

18. As described in Wei II, treatment of plants with the hypersensitive response elicitor HrpN from *Erwinia amylovora* resulted in disease resistance to a broad range of plant pathogens. For example, HrpN induced disease resistance to southern bacterial wilt (*Pseudomonas solanacearum*) in tomato, tobacco mosaic virus in tobacco, and bacterial leaf spot (*Gliocladium cucurbitae*) in cucumber.

19. The hypersensitive response elicitor HrpZ from *Pseudomonas syringae* was reported to induce disease resistance in cucumber to a diverse range of pathogens, including the fungal disease *Colletotrichum lagenarium*, tobacco necrosis virus, and bacterial angular leaf spot (*Pseudomonas syringae* pv. *lachrymans*). See Strobel et al., "Induction of Systemic Acquired Resistance in Cucumber by *Pseudomonas syringae* pv. *syringae* 61 $HrpZ_{Pss}$ Protein," Plant Journal 9(4): 431-439 (1996) ("Strobel") (attached hereto as **Exhibit 14**).

20. Hypersensitive response elicitors from *Erwinia amylovora* and *Pseudomonas syringae* pv. *syringae* are also known to enhance plant growth. See Examples 1 to 24 of U.S. Patent No. 6,277,814 to Qiu et al. ("Qiu") (attached hereto as **Exhibit 15**), which showed that treatment of plants and plant seeds with HrpN from *E. amylovora* induced plant growth enhancement in species of tomato, potato, raspberry, and cucumber.

Hypersensitive Response Elicitors Induce Plant Disease Resistance

21. As demonstrated by the following experimental evidence in paragraphs 22 and 23 below, treatment of tomato and tobacco plants with the hypersensitive response elicitor HreX from *Xanthomonas campestris* pv. *pelargonii* induced disease resistance in the plants against bacterial wilt and tobacco mosaic virus.

22. The induction of disease resistance in tomato against bacterial wilt (caused by the pathogenic bacterium *Pseudomonas solanacearum* K_{60}) was investigated as follows. Approximately 30 days after sowing, tomato plants were sprayed with either a dilution of HreX or 5 mM potassium phosphate buffer, pH 6.8 (the same buffer used to dilute the HreX solution). Six days after treatment, inoculation was performed by slicing the soil of the pot containing the tomato plant 4 times and applying 40 ml of solution containing 1×10^6 colony forming units ("cfu") per ml of *P. solanacearum* K_{60} to the soil. Disease severity ratings were recorded at 7, 9, and 13 days after inoculation ("DAI"), as shown below in Table 1. As these results demonstrate, plants treated with HreX exhibited substantially more disease resistance than the buffer-treated control plants.

Table 1. ***Pseudomonas solanacearum* Disease Resistance from Treatment of Tomato with HreX.**

Treatment Groups[a]	Disease Index (7 DAI)	Disease Index (9 DAI)	Disease Index (12 DAI)	% Difference (12 DAI)
HreX	0.12	0.22	0.22	38.89
Buffer	0.16	0.3	0.36	na

[a]Each group consisted of 1 pot containing 10 plants.

23. Experiments examining the induction of systemic disease resistance in tobacco from treatment with HreX were conducted as follows: Diluted HreX was sprayed on all but the bottom most full-sized leaf of six- to eight-week-old tobacco plants (Xanthi). The bottom most full-sized leaf was covered during spraying so as not to receive residual spray. Three days after the spray treatment, the unsprayed leaf and the leaf opposite it, were lightly

dusted with diatomaceous earth. Thereafter, 20 μl of a 1.7 μg/ml solution of tobacco mosaic virus ("TMV") was applied to both leaves dusted with diatomaceous earth. The TMV was gently and evenly spread across the leaves. Approximately 5 minutes after inoculation, the plants were lightly rinsed to remove the diatomaceous earth. Three days after inoculation, the number of TMV lesions on the unsprayed and sprayed leaves for each plant was recorded, as shown below in Table 2. As these results demonstrate, plants treated with HreX exhibited substantially more disease resistance than the buffer-treated control plants.

Table 2. Tobacco Mosaic Virus Resistance in Tobacco from Treatment with HreX.

Treatment Groups	Number of TMV Lesions on Leaf									
	Treated leaves					Untreated leaves				
	Plant No. 1	Plant No. 2	Plant No. 3	Avg. No.	% Difference	Plant No. 1	Plant No. 2	Plant No. 3	Avg. No.	% Difference
HreX	5	7	8	6.67a	93.37	41	22	20	27.67a	76.49
Buffer Control	107	99	96	100.67b	na	124	106	123	117.67b	na

Enhanced Plant Growth by Treatment of Plants with Hypersensitive Response Elicitor

24. As demonstrated by the following experimental evidence in paragraphs 25 and 26 below, treatment of plants with hypersensitive response elicitors from a range of sources, such as *Psuedomonas syringae* (HrpZ) and *Xanthomonas campestris* (HreX), enhances plant growth.

25. The hypersensitive response elicitor HreX from *Xanthomonas campestris* was evaluated for induction of plant growth enhancement as follows: Prior to sowing, tomato seeds were soaked for approximately four hours in either a solution containing the partially purified HreX protein diluted in potassium-phosphate buffer, or potassium-phosphate buffer alone. The treated seeds were then planted and maintained in identical conditions in a controlled environment. Each treatment group consisted of 3 pots, each pot containing 8 plants. The average plant heights and percent differences between the treatment groups are shown below in Table 3. As these results demonstrate, plants treated with HreX grew significantly more than the buffer-treated control plants.

Table 3. Growth Enhancement from Treatment of Tomato with the Hypersensitive Response Elicitor HreX.

Treatment Groups	Replicates[1]			Mean[2]	% Difference
	Pot #1	Pot #2	Pot #3		
HreX	7.4	7.3	6.8	7.1a	15.5
Buffer Control	6.1	6.1	5.6	6.0b	na

[1] Mean height of the 8 plants in each pot.
[2] Means followed by the same letter do not significantly different (P=0.01, LSD)

26. The hypersensitive response elicitor HrpZ from *Psuedomonas syringae* was evaluated for induction of plant growth enhancement as follows: Prior to sowing, tomato seeds were soaked for approximately four hours in either a solution containing the partially purified HrpZ protein diluted in potassium-phosphate buffer, or potassium-phosphate buffer alone. The treated seeds were then planted and maintained in identical conditions in a controlled environment. Each treatment group consisted of 6 pots, each pot containing 10 plants. The average plant heights and percent differences between the treatment groups are shown below in Table 4. As these results demonstrate, plants treated with HrpZ grew significantly more than the buffer-treated control plants.

Table 4. Growth Enhancement from Treatment of Tomato with the Hypersensitive Response Elicitor HrpZ.

Treatment Groups	Replicates[1]						Mean[2]	% Difference
	Pot #1	Pot #2	Pot #3	Pot #4	Pot #5	Pot #6		
HrpZ	5.10	5.28	4.60	4.72	4.71	4.87	4.88a	9.6
Buffer Control	4.15	4.38	3.84	4.31	4.62	5.18	4.41b	na

[1] Mean height of the 18 to 21 plants in each pot.
[2] Means followed by the same letter do not significantly differ (P=0.054, LSD)

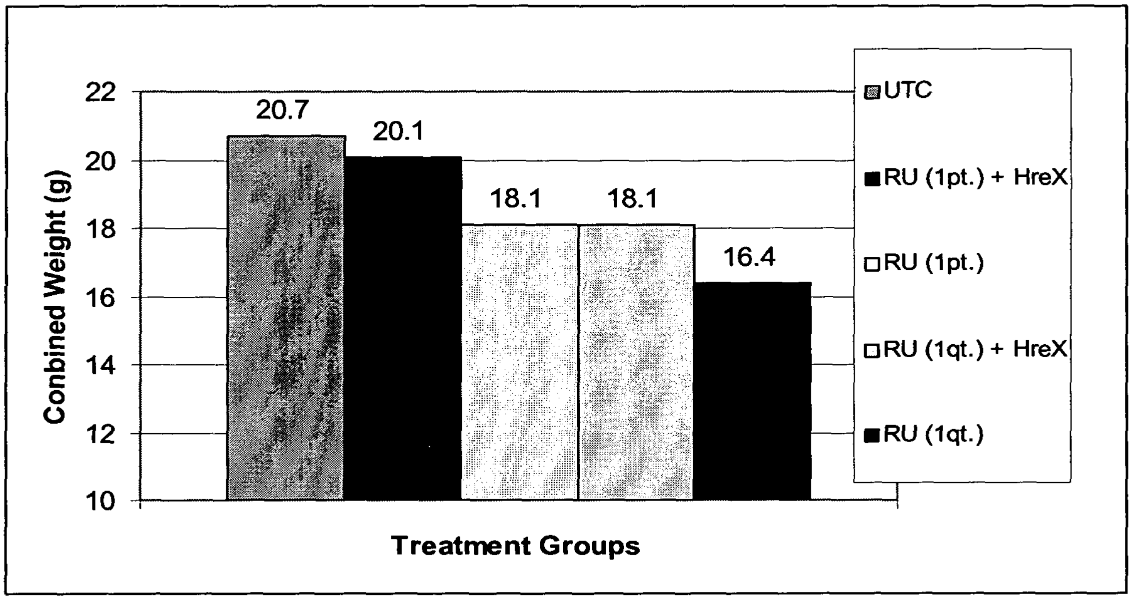
Hypersensitive Response Elicitors Induce Plant Stress Resistance

27. As evidenced by the experimental results reported in Example 12 of the above-identified application and Examples 1-6 of WO 00/28055 to Wei et al. (attached hereto as **Exhibit 16**), HrpN from *Erwinia amylovora* is capable of inducing various forms of plant stress resistance, such as chemical stress resistance, drought stress resistance, and nutritional stress resistance.

28. As demonstrated by the following experimental evidence in paragraphs 29 through 32 below, the hypersensitive response elicitor HreX from *Xanthomonas campestris* is also capable of inducing various forms of plant stress resistance, such as chemical stress resistance and salt stress resistance.

29. In order to investigate whether treatment of plants with the hypersensitive response elicitor HreX from *Xanthomonas campestris* induces chemical stress resistance, corn seeds (DK662RR) were treated with HreX and then treated with varying concentrations of Roundup® (active ingredient glyphosate, Monsanto Co., St. Louis, MO). The HreX treatments consisted of soaking the corn seeds in 100 ml of a solution containing a 3% formulation of HreX dissolved in water. The seeds were soaked for approximately 4 hours at 26°C. The corn seeds were sown in pots containing vermiculite and equal amounts of the fertilizer EcoGrow (ECO Enterprises, Shoreline, WA). Roundup® (RU) treatments were conducted by a single spraying of the corn seedlings approximately two weeks after germination. Roundup® was applied at two concentrations. At the 1 pint (1pt.) application rate, 4.73 ml of Roundup® was mixed with 189 ml water. At the 1 quart (1qt.) application rate, 9.46 ml of Roundup® was mixed with 189 ml of water. The specific treatment groups were as detailed below in Figure 1. Fifteen seeds were planted in each pot with a total of six pots per treatment. Plants were grown at 22°C to 26°C with a 14 hour daylight period. Results were obtained by measuring the dry weight from the largest 10 plants from each pot. The plants were dried by isolating the entire plant from the vermiculate and drying overnight at 26°C. The Combined Weight shown below in Figure 1 represents the accumulated dry weight of the 60 plants measured from each treatment group. The untreated control (UTC) plants were not pretreated with HreX and were not treated with Roundup®.

Figure 1. Dry Weight of Chemical + HreX and Chemical Alone Treated Plants

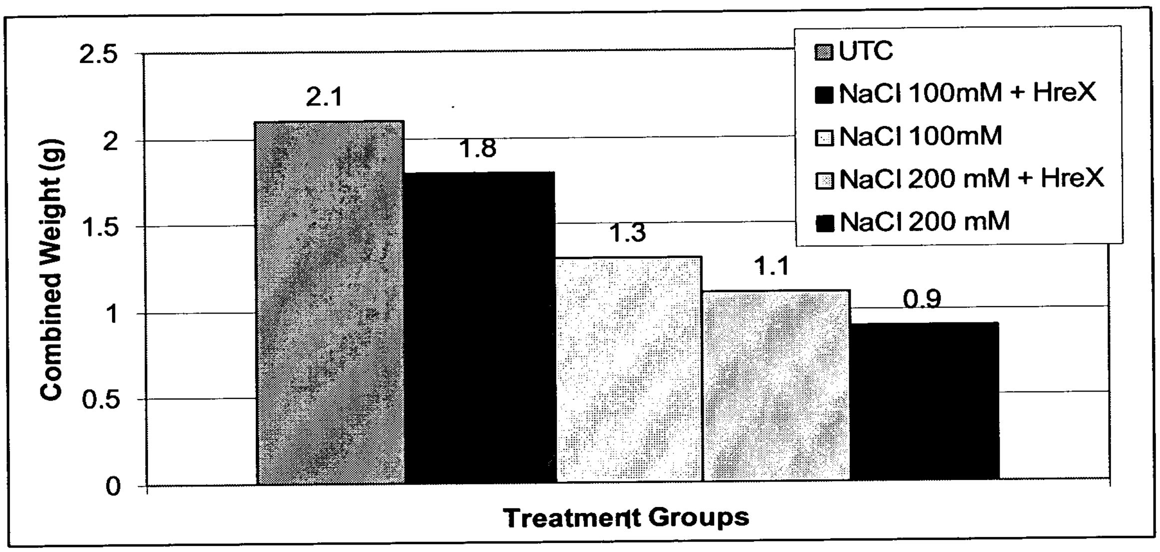


30. The hypersensitive response elicitor HreX clearly imparts chemical stress resistance in plants as demonstrated in Figure 1. Treatment of plants with Roundup® led to decreases in plant dry weight of approximately 13% at the Roundup® application rate of 1 pint, and approximately 21% at the Roundup® application rate of 1 quart, in comparison to that of the untreated control plants. In contrast, plants treated with HreX in combination with Roundup® resulted in decreases in dry weight of approximately 3% at the Roundup® application rate of 1 pint, and approximately 13% at the Roundup® application rate of 1 quart, in comparison to that of the untreated control plants. The treatment of plants with the hypersensitive response elicitor HreX increased the growth of Roundup® treated plants by 9 to 10%.

31. In order to investigate whether treatment of plants with the hypersensitive response elicitor HreX from *Xanthomonas campestris* imparts salt stress resistance in plants, lima bean seeds (Dixie Speckled Peas) were treated with HreX, sown, and then maintained in the presence of varying concentrations of NaCl. HreX treatment consisted of soaking the seeds in 100 ml of a solution containing a 3% formulation of HreX dissolved in water. The seeds were soaked for approximately 4 hours at 26°C. The lima beans were grown in pots containing vermiculite, equal amounts of the fertilizer EcoGrow (ECO Enterprises, Shoreline, WA), and varying concentrations of NaCl. The treatment groups were as detailed below in Figure 2. Fifteen seeds were planted in each pot, with a

total of six pots per treatment. Plants were grown at 22°C to 26°C with a 14 hour daylight period. Results were obtained by measuring the dry weight from the largest 10 plants from each pot. The plants were dried by isolating the entire plant from the vermiculate and drying overnight at 26°C. The Combined Weights detailed in Figure 2 below represent the accumulated dry weight of the 60 plants measured from each treatment. The untreated control (UTC) plants were not treated with HreX and were not grown in the presence of NaCl. The results of the study are shown below in Figure 2.

Figure 2. Dry Weight of Salt + HreX and Salt Alone Treated Plants



32. The hypersensitive response elicitor HreX clearly imparts salt stress resistance in plants, as demonstrated in Figure 2. Growth of the plants in the presence of 100 mM and 200 mM NaCl resulted in decreases in plant dry weight of approximately 38% and 57%, respectively, in comparison to that of the untreated control plants. In contrast, plants treated with HreX and grown in the presence of 100 mM and 200 mM NaCl resulted in decreases in plant dry weight of approximately 14% and 48%, respectively, in comparison to that of the untreated control plants. The treatment of plants growing in the presence of high concentrations of NaCl with the hypersensitive response elicitor HreX resulted in increases in plant dry weight of 18 to 28%.

33. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: Feb. 3/03

Zhong-Min Wei

JB FYI Poms

Suresh Gopalan and Sh ng Yang H
MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing

Bacterial Genes Involved in the Elicitation of Hypersensitive Response and Pathogenesis

Intensive molecular genetic studies undertaken in the past 10 years have started to solve many of the puzzles in the area of compatibility and incompatibility between plants and bacterial pathogens. These studies have provided answers to some of the most fundamental questions in plant pathology: What bacterial genes are involved in the establishment of compatibility or incompatibility between plants and necrogenic bacteria? What traits distinguish plant-pathogenic bacteria from saprophytic bacteria? Are these genes and traits common in seemingly very diverse groups of plant-pathogenic bacteria, from soft-rot erwinias to local lesion-forming pseudomonads? In this article, we will discuss some recent advances in understanding the compatibility or incompatibility between plants and necrogenic bacteria (bacteria that cause tissue necrosis). The potential application of these advances to disease management will be addressed briefly. Interested readers should consult other recent reviews (6,8,45,50) for a more technical discussion on this topic.

Plant–Bacteria Interactions: Incompatible vs. Compatible

Plant-pathogenic bacteria cause devastating diseases on many important crop plants. Some bacteria, such as *Agrobacterium tumefaciens*, cause tissue deformation (tumors) by altering hormone balance in infected plant tissues. Other bacteria cause wilt or soft rot by interfering with the function of the plant vascular system or by disintegrating plant tissues, respectively. Many pathovars of *Pseudomonas syringae* and *Xanthomonas campestris* cause local lesions on various plant tissues. Disease symptoms caused by most plant-pathogenic bacteria involve plant cell death. In this article, only necrogenic bacteria will be discussed. Therefore, gall-forming *A. tumefaciens* and other bacteria that do not cause necrosis will not be addressed.

Plant–bacteria interactions can be generally classified as compatible or incompatible interactions. In a compatible interaction, a susceptible host plant is infected by a virulent (or compatible) bacterium, resulting in the multiplication and spread of the bacterium in infected plant tissues and the appearance of disease symptoms. In an incompatible interaction, an avirulent (or incompatible) bacterium attempts to infect a resistant host plant or a nonhost plant, but the multiplication and spread of the bacterium are severely restricted. A hallmark of many incompatible interactions is the occurrence of rapid plant cell death at or near the attempted infection site, a phenomenon known as the hypersensitive response (HR; 16,29). That is, although an avirulent bacterium is unable to cause typical spreading disease symptoms in a resistant host or nonhost plant, it is able to elicit localized plant cell death. The HR is associated with a wide array of defense responses that may inhibit further pathogen invasion, including synthesis of antimicrobial compounds, induction of plant defense genes, and strengthening of the plant cell wall by rapid cross-linking of cell wall components (10,32).

Although a true plant-pathogenic bacterium can elicit a dramatic plant response—either disease or resistance—in a healthy plant with the appropriate genetic background, saprophytic bacteria or bacteria that are pathogenic on organisms other than higher plants are generally unable to initiate any interactions in plants. Of 1,600 known species of bacteria, only about 80 species have been found to cause plant disease (1). What are the features that distinguish plant-pathogenic bacteria from other types of bacteria? Taxonomic differences give no clue to the differences in pathogenicity. For example, *Erwinia amylovora*, the bacterium that causes fire blight, is taxonomically more closely related to the human pathogens *Escherichia coli* and *Yersinia* spp. than to another common plant pathogen *P. syringae*.

Genes Controlling Compatibility Between Plants and Bacteria

In the early 1980s, a number of researchers started to use transposon-mediated mutagenesis, a technique developed in the study of *E. coli*, to reveal bacterial genes that play important roles in various plant–bacteria interactions. A transposon is a mobile DNA element that can hop in and out of the bacterial chromosome. When a transposon hops into a gene on the chromosome, the gene is physically disrupted and cannot produce a functional product (Fig. 1). If the gene happens to be important in plant–bacterial interactions, the mutant bacterium carrying the disrupted gene will show a defect in initiating normal plant–bacterial interactions.

Using such a mutagenesis technique, Niepold et al. (35) and Lindgren et al. (33) identified clusters of bacterial genes, known as *hrp* (for HR and pathogenicity) genes, in the bean pathogens *Pseudomonas syringae* pv. *syringae* and *P. s.* pv. *phaseolicola*, respectively. Transposon-induced mutations in *hrp* genes were found to abolish the ability of *P. syringae* to elicit the HR in nonhost plants or to cause disease in host plants (33,35). *hrp* mutants behave very much like bacteria that have no apparent interactions with plants, such as *E. coli*. The identification of *hrp* genes suggested that the molecular mechanism(s) underlying bacterial pathogenicity and bacterial elicitation of plant disease resistance may involve the same bacterial genes.

hrp genes have been isolated from many plant-pathogenic bacteria and have been characterized most extensively from *P. s.* pv. *syringae*, *P. s.* pv. *phaseolicola*, *Pseudomonas solanacearum* (which causes wilt in many solanaceous plants), *Xanthomonas campestris* pv. *vesicatoria* (which causes bacterial spot on tomato and pepper), and *E. amylovora* (6,8,45). Isolation (cloning) of *hrp* genes was accomplished by inserting random genomic DNA fragments from a wild-type, plant-pathogenic bacterium into a cloning vector, followed by introduction of cloned DNA fragments into *hrp* mutants

Dr. He's address is: MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824-1312; Phone: 517-353-9181, Fax: 517-353-9168, E-mail: hes@pilot.msu.edu

Publication no. D-1996-0313-04F

(Fig. 1). If a cloned DNA fragment carries a wild-type copy of the mutated *hrp* gene in an *hrp* mutant, it will produce a functional *hrp* gene product and therefore complement the mutated *hrp* gene located in the chromosome (Fig. 1). Surprisingly, the cloned *hrp* clusters from *P. s.* pv. *syringae* 61 and *E. amylovora* 321 enabled nonpathogens (e.g., *E. coli* or *Pseudomonas fluorescens*) to elicit the HR in plants (5,24). The functional cloning of these two *hrp* clusters in *E. coli* revealed that the minimum number of genes required for elicitation of the HR by plant-pathogenic bacteria is carried on a DNA fragment about 25 to 30 kb in length, a very small portion of the bacterial genome (which is normally about 4,000 to 5,000 kb).

DNA–DNA hybridization studies indicate that at least some *hrp* genes are similar among necrogenic bacteria belonging to different genera (*P. syringae*, *E. amylovora*, *Erwinia stewartii*, *P. solanacearum*, and *X. campestris*) (31). Recent DNA sequence studies confirm that many *hrp* genes cloned from diverse plant-pathogenic bacteria are homologous (23,46). Thus, *hrp* genes appear to be universal among diverse necrosis-causing, gram-negative bacterial pathogens of plants.

Biochemical Functions of *hrp* Genes

The biochemical functions of *hrp* genes have remained a puzzle until recently. DNA sequencing has played a major role in the determination of many *hrp* gene functions. As will be discussed, many *hrp* genes have striking similarities with genes of known function. Figure 2 shows the gene organization and likely functions of *hrp* genes of *P. s.* pv. *syringae* (23). There are at least 25 *hrp* genes in this bacterium. Based on DNA sequence similarity to other known genes and subsequent biochemical and molecular characterization, we now know that *hrp* genes have at least three biochemical functions: gene regulation, protein secretion, and production of HR elicitor proteins.

1. Gene regulation. It was discovered that *hrp* genes either are not expressed or are expressed at very low levels (i.e., they make very low levels of protein products) when bacteria were grown in nutrient-rich bacteriological media, whereas they are highly expressed when bacteria are in the intercellular space (apoplast) of plant tissues (25,37,41,46,48,52,53). What conditions in plant tissues induce the expression of *hrp* genes, and how do bacteria detect these inducing conditions? Unlike viruses, nematodes, and many fungi, plant-pathogenic bacteria do not invade living plant cells. Therefore, signal exchanges between plant cells and bacteria must occur in (or through) the apoplast outside the plant cell. A number of laboratories have observed that induction of *P. syringae hrp* genes could be achieved by using artificial minimal media lacking complex nitrogen nutrients, indicating that lack of nutrients in the plant apoplast may be the signal for the induction of *hrp* genes (25,37,52,53). Specific compounds (e.g., sucrose and sulfur-containing amino acids) present in the plant apoplast may also serve as signals for the induction of *X. c.* pv. *vesicatoria hrp* genes (41). The induction of *hrp* genes in the nutrient-poor plant apoplast or in artificial minimal media indicates that *hrp* genes may be involved in bacterial nutrition in planta.

How do bacteria sense the plant apoplast environment? It was found that at least three of the 25 *hrp* gene products are involved in detection of the apoplast environment by *P. syringae*: *hrpL*, *hrpS*, and *hrpR* (18,51; Fig. 2). The *hrpS* and *hrpR* are among the first two *hrp* genes to be expressed once bacteria enter plant tissues (51,52). It has been hypothesized that the HrpS and HrpR proteins, once produced, bind to the "promoter" sequence of the *hrpL* gene to "promote" the production of the *HrpL* protein (51). Once the HrpL protein is produced, it activates promoters of other *hrp* genes and some bacterial avirulence (*avr*) genes, which determine gene-for-gene interactions between bacteria and plants (25,26,38,40,51; Fig. 3). Not all bacterial *avr* genes are regulated by *hrp* genes (30). Interestingly, *hrpS* and *hrpR* are similar in sequence to a family of bacterial proteins that regulate genes involved in diverse metabolic functions, including genes involved in nutrient transport and metabolism (18,51). The sequence similarity of *hrpS* and *hrpR* with gene regulators involved in nutrition appears to support the hypothesis that *hrp* genes are involved in bacterial nutrition in the nutrient-poor plant apoplast. This hypothesis is further supported by the observation that the expression of *hrp* genes can be turned off by complex nitrogen sources, tricarboxylic acid cycle intermediates, high osmolarity, and neutral pH, some of which are characteristic of rich bacterial media (25,37,41,46,52,53).

An *hrpS* homolog has been found in a very different bacterium, *E. amylovora* (S. V. Beer, *personal communication*). In *P. solanacearum*, a different *hrp* gene (*hrpB*) was found to be involved in the detection of the plant apoplast (15). Thus, different bacteria may or may not use the same mechanism to detect the apparently similar environment in the plant apoplast.

2. Protein secretion. One surprising finding from the sequence analysis of *hrp* genes was that many *hrp* genes show striking similarities to those involved in the secretion of proteinaceous virulence factors in human and animal pathogenic bacteria (12,17,22,39,46). Most plant-pathogenic

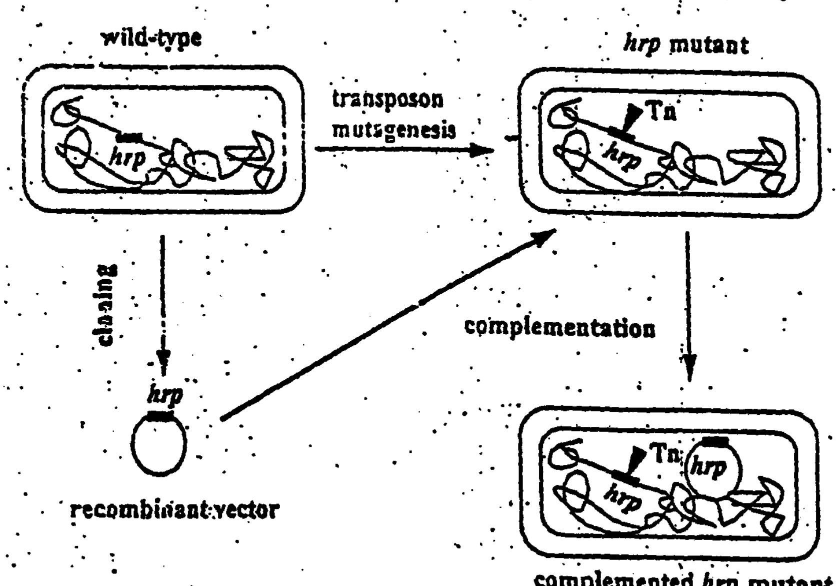


Fig. 1. Diagram of molecular techniques commonly used in the cloning of *hrp* genes. A wild-type bacterium is mutagenized by random insertion of a transposon (Tn) into its genome. When a transposon inserts into a wild-type *hrp* gene (in red), it physically disrupts the *hrp* gene (in green). The transposon-inserted *hrp* gene cannot produce a functional product, and the resulting bacterium is called a *hrp* mutant. The *hrp* mutant can no longer induce the hypersensitive response (HR) in resistant plants or cause disease in susceptible plants. To isolate (clone) the *hrp* gene identified by transposon mutagenesis, a gene library is established by inserting pieces of the wild-type genomic DNA into a cloning vector (indicated by a circle). The vector carrying foreign DNA inserts (recombinant vector) is then introduced into the *hrp* mutant. If a recombinant vector happens to carry a wild-type copy of the mutated *hrp* gene, it will produce a functional *hrp* gene product lacking in the *hrp* mutant, thus recovering the ability of the mutant to induce the HR in resistant plants and to cause disease in susceptible plants. The *hrp* mutant phenotype is therefore complemented by this recombinant vector.

bacteria that cause necrosis are gram-negative, that is, they have two cell membranes enveloping the cytoplasm. For any large molecule (e.g., a protein) to go through a lipid membrane, a special secretion apparatus or channel composed of many proteins must be assembled across both cell membranes. Gram-negative plant pathogenic bacteria are known to make several types of secretion apparatus. For example, *Erwinia chrysanthemi*, a bacterium that causes soft rot, makes one type of secretion apparatus for proteases and another for plant cell wall-degrading enzymes (21,39). Both types of secretion apparatus are widely conserved among many other bacteria, including human pathogens such as *E. coli* and *Pseudomonas aeruginosa* (21,39). The *hrp* genes were found to specify a third type of secretion apparatus, the Hrp secretion apparatus, which appears to be similar to the one discovered in several human-pathogenic bacteria, including *Yersinia* spp. (12,17,22,39,46). Interestingly, although the regulatory *hrp* genes in different bacteria may be different (*hrpS*, *hrpR*, and *hrpL* in *P. syringae* versus *hrpB* in *P. solanacearum*), most *hrp* genes involved in the assembly of the Hrp secretion apparatus are similar among diverse plant-pathogenic bacteria. This suggests that although different bacteria may detect the plant apoplast environment in their own unique ways, they nevertheless produce similar types of protein secretion apparatus.

3. Production of elicitor proteins. The discovery of the novel Hrp secretion apparatus raised an immediate question: What are the proteins that traverse it? Since *hrp* genes are essential for bacteria, both to elicit the plant HR and to cause disease, it was expected that some of the proteins that traverse the Hrp secretion apparatus may be elicitors of plant HR and that others may be involved in causing necrosis during pathogenesis. Wei et al. (47) first provided evidence that one of the *E. amylovora hrp* genes (*hrpN*) encodes a proteinaceous elicitor (harpin). Harpin elicits HR necrosis when injected into the apoplast of appropriate plants (47). Although no *hrpN* gene homolog could be found in *P. syringae*, another proteinaceous HR elicitor (harpin$_{Pss}$) was identified and was shown to be encoded by a different *hrp* gene, *hrpZ* (20,36). Furthermore, harpin$_{Pss}$ was the first extracellular protein shown to be secreted via the Hrp secretion apparatus (20). A third bacterial protein elicitor of the HR was identified in *P. solanacearum* and was named PopA1 (2). The *E. amylovora* harpin, *P. s.* pv. *syringae* 61 harpin$_{Pss}$, and *P. solanacearum* PopA1, although largely dissimilar in primary sequences, share similar properties that may be important in their HR elicitor activities. They are all heat stable, glycine rich, and hydrophilic. Homologs of *E. amylovora* harpin and *P. s.* pv. *syringae* 61 harpin$_{Pss}$ have been identified in other pathogenic strains that belong to the genus *Erwinia* and the species *P. syringae*, respectively (4,20). Thus, at least three proteins that traverse the Hrp secretion apparatus of three diverse bacteria elicit the HR.

The Search for Proteins that Traverse the Hrp Apparatus

As mentioned earlier, bacterial mutants defective in the Hrp secretion apparatus are unable to elicit the HR in resistant plants and to cause disease in susceptible plants. The question is, how many proteins are secreted via the Hrp secretion apparatus? If harpins and PopA are the only proteins that traverse the Hrp secretion apparatus, then mutations in the genes that make harpins and PopA would also eliminate the ability of bacteria to elicit the HR in resistant plants and to cause disease in host plants. However, if there are other pathogenicity- or HR-related proteins secreted via the Hrp apparatus, mutations in only harpin- or PopA-encoding genes would not completely abolish the ability of bacteria to elicit the HR in resistant plants or to cause disease in host plants. Wei et al. (47) reported that mutations in the gene coding for harpin of *E. amylovora* destroyed the ability of the bacteria both to trigger the HR in resistant nonhost tobacco and to cause disease in susceptible pear fruits. Mutations in the gene coding for harpin$_{Ech}$ of *E. chrysanthemi* prevented the bacterium from triggering the HR in the nonhost tobacco and reduced the ability of the bacterium to initiate disease lesions in host plants (4). In the case of harpin$_{Pss}$ of *P. syringae*, mutation analysis has been complicated by the complex gene structure and organization surrounding the *hrpZ* gene. Conclusive data regarding the role of harpin$_{Pss}$ in plant–*P. syringae* interactions are yet to be shown. PopA1 was shown to

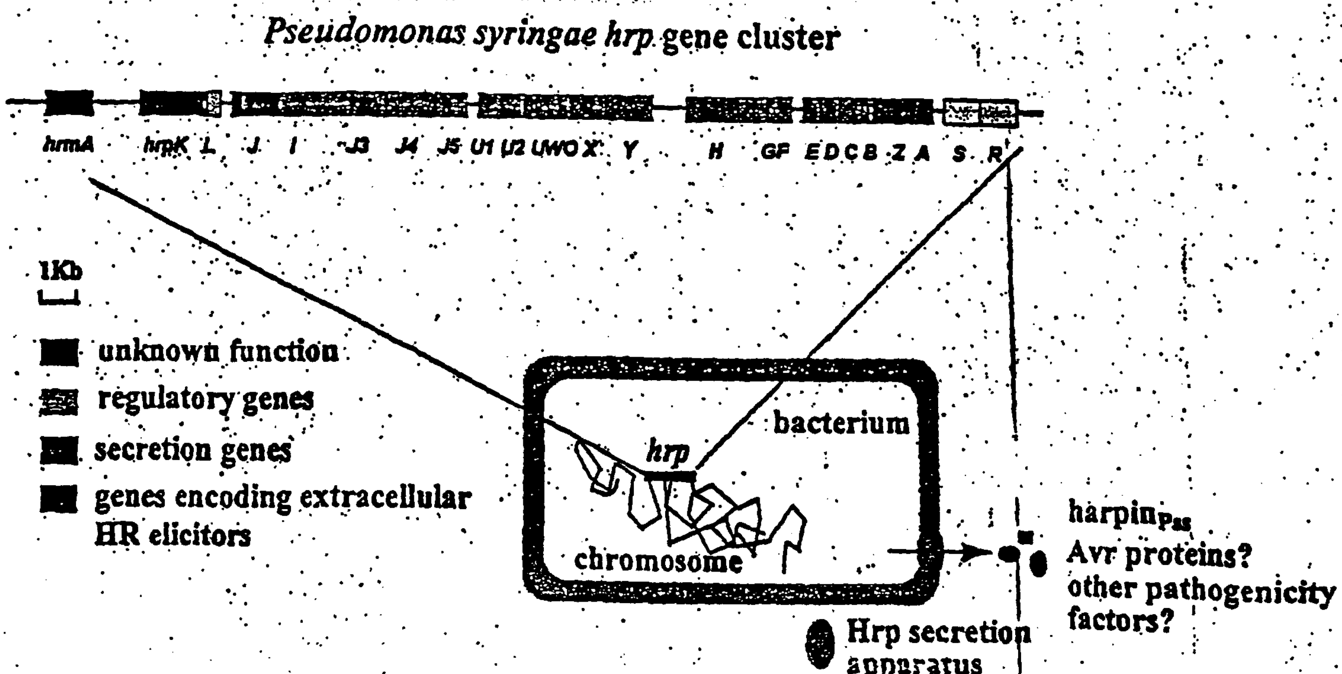


Fig. 2. *hrp* genes of *Pseudomonas syringae*. There are at least 25 *hrp* genes (*hrpA* to *hrpZ*) in *P. syringae*. *hrpS*, *hrpR*, and *hrpL* (in yellow) are involved in the detection of the plant apoplast environment and in the activation of all other *hrp* genes, *avr* genes, and possibly other pathogenicity-related genes. Most other *hrp* genes (in red) are involved in the assembly of the Hrp secretion apparatus in the bacterial envelope, through which travels a newly discovered class of bacterial virulence/avirulence proteins (in green), including HrpZ.

be dispensable for pathogenicity of *P. solanacearum* in the susceptible host plant, tomato, or for HR elicitation in the nonhost plant, tobacco (2), indicating that there must be other HR-elicitors and pathogenicity factors that traverse the Hrp secretion apparatus in this bacterium. Further examination indicated that PopA1 may function as an avirulence gene, mediating gene-for-gene interaction in certain Petunia–*P. solanacearum* interactions (2,45). Thus, the Hrp secretion apparatus in each bacterium may secrete a different number of proteins. Identification of other proteins that traverse the Hrp secretion apparatus is now an active research area and is essential for a complete understanding of *hrp*-mediated plant–bacterial interactions.

Factors Modifying *hrp* Gene-Mediated Compatibility

Two broad classes of bacterial genes may superimpose their functions on the *hrp* gene-mediated compatibility or incompatibility between plants and bacteria: *avr* genes and various virulence genes. The *avr* genes mediate genotype-specific incompatibility in resistant host plants. Virulence genes promote the production of disease symptoms and are usually needed for the full virulence of bacteria.

Bacterial *avr* Genes

Flor (14) formulated the gene-for-gene hypothesis in his work on flax–flax rust interactions. Flor hypothesized that the resistance of a given flax cultivar to a given fungal race is the result of the interaction between a fungal *avr* gene and a corresponding flax resistance gene. Therefore, the interactions between the plant's resistance genes and the pathogen's *avr* genes would limit the host range of the pathogen. Staskawicz et al. (44) first cloned an *avr* gene from a soybean bacterial pathogen, *Pseudomonas syringae* pv. *glycinea*, and showed that the cloned *avr* gene could convert virulent *P. s.* pv. *glycinea* strains that cause disease into avirulent strains that elicit the HR in certain soybean cultivars carrying the corresponding resistance genes, thus validating the role of *avr* genes in controlling host range. Since then, numerous *avr* genes have been cloned from plant-pathogenic bacteria (27). Several plant resistance genes have also been cloned using molecular genetic approaches (e.g., 34,43).

What is the relationship between the *avr* genes and *hrp* genes, both of which are involved in eliciting the HR? Several laboratories have observed that *avr* genes cannot trigger the genotype-specific HR in *hrp* mutants, i.e., *avr* genes depend on functional *hrp* genes for expressing their phenotype (25,26,28,38,40). There are several ways of explaining such dependence (Fig. 4). One possibility is that Avr proteins are dependent on the Hrp secretion apparatus for secretion. Alternatively, Avr function requires a prior plant response elicited by the *hrp*-controlled extracellular factors (such as harpins). A third possibility is that Avr proteins, with no HR-eliciting activity by themselves, cause the cultivar-specific HR by either covalently modifying harpins or modulating the expression of harpins in a plant resistance gene-dependent manner yet to be understood. Finally, it is also possible that Avr proteins are secreted directly into the plant cell with the help of harpins, assuming that receptors for Avr proteins are inside the plant cell. Studies are being carried out to resolve these possibilities.

Bacterial Virulence Factors

The genetic diversity of plant-pathogenic bacteria is reflected in their ability to cause diverse disease symptoms ranging from soft rot to tissue necrosis to "wildfire." These diverse disease symptoms are likely the result of the action of several, sometimes unique, virulence factors produced by a given bacterium in addition to *hrp*-controlled pathogenicity factors. For example, research from many laboratories has shown that toxin production plays an important role in the formation of chlorosis and necrosis (3,19,49). Extracellular polysaccharides may be involved in the formation of water-soaking lesions (11,13) and in the production of wilt symptoms by clogging the plant vascular system (9). Plant cell wall–degrading enzymes are responsible for tissue disintegration and the appearance of the soft-rot symptom (7). Plant hormones produced by plant-pathogenic bacteria are involved in the induction of tissue deformation (42).

Both *hrp* genes and bacterial virulence factors are necessary for disease symptom production, but what is the relationship between them? A logical relationship would be that *hrp*-controlled extracellular factors are involved in obtaining nutrients in early stages of pathogenesis, whereas other virulence factors drive the initial compatible stage into a fully compatible one, leading to the production of various disease symptoms. At least two lines of

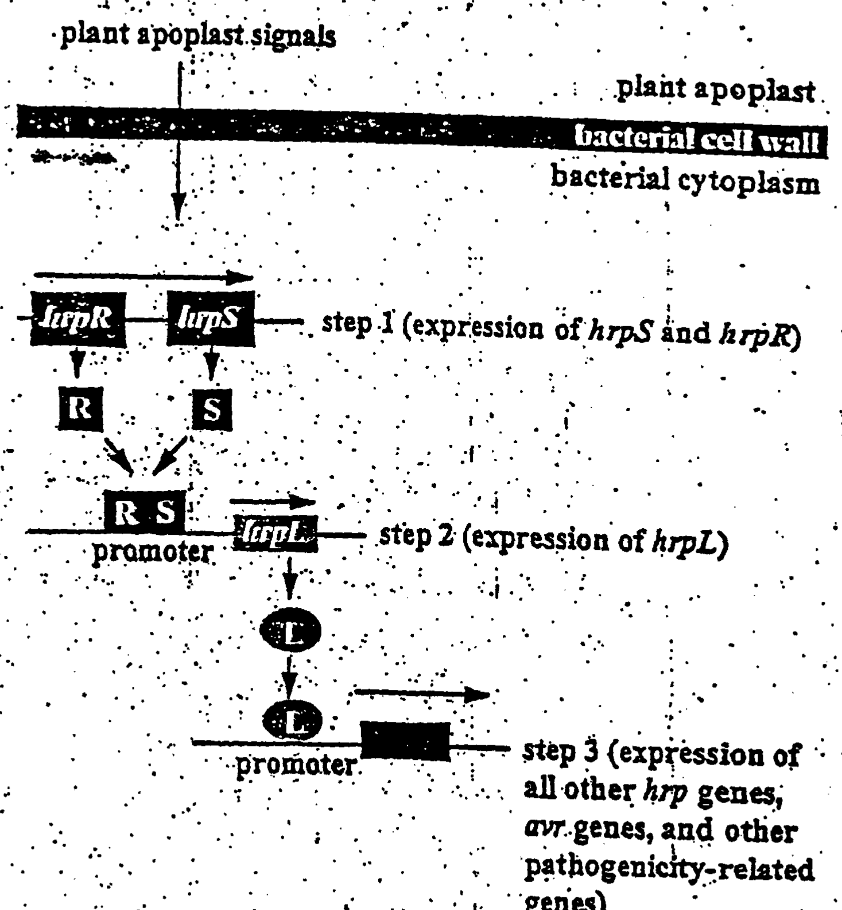


Fig. 3. Diagram of the signal transduction cascade in the detection of the plant apoplast environment by *Pseudomonas syringae*. The plant ap plast environment (limited nutrients and/or certain uniqu c mpounds) activates the expression of *hrpS* and *hrpR* by a mechanism yet to be understood (step 1). The *hrpS* and *hrpR* gene pr ducts (S and R, respectively) bind to and activate the promoter of the *hrpL* gen (step 2). The *hrpL* gene product (L), in turn, binds to prom ters of ther *hrp* genes, *avr* genes, and other bacterial pathogenicity-related gen s to promote th expression of these genes, resulting in the initiation of diverse plant–bacteria interactions (step 3). Modified from Xiao et al. (51).

evidence seem to support this relationship. First, *hrp* genes are highly conserved among diverse plant-pathogenic bacteria, whereas virulence factors vary greatly among bacteria. Second, while mutations in the *hrp* gene completely abolish both bacterial pathogenicity and elicitation of the HR, mutations in virulence genes (e.g., toxin-production genes) often do not eliminate pathogenicity and have no effect on bacterial elicitation of the HR (3,19,49).

hrp Gene Functions and Disease Management

A major reason for discovering bacterial and plant factors critical for bacterial pathogenesis and plant resistance is to develop novel and environmentally safe strategies for controlling plant diseases. The discovery that the Hrp secretion apparatus is crucial to bacterial pathogenesis provides a foundation for designing novel chemicals and antibodies that would block the assembly of the Hrp secretion apparatus or the passage of bacterial virulence proteins through it. Alternatively, susceptible crop plants could be genetically engineered with genes encoding proteinaceous HR elicitors, such as harpins, under the control of plant promoters inducible by virulent pathogens. If this approach were successful, the HR or resistance would be triggered in otherwise compatible interactions, limiting disease development.

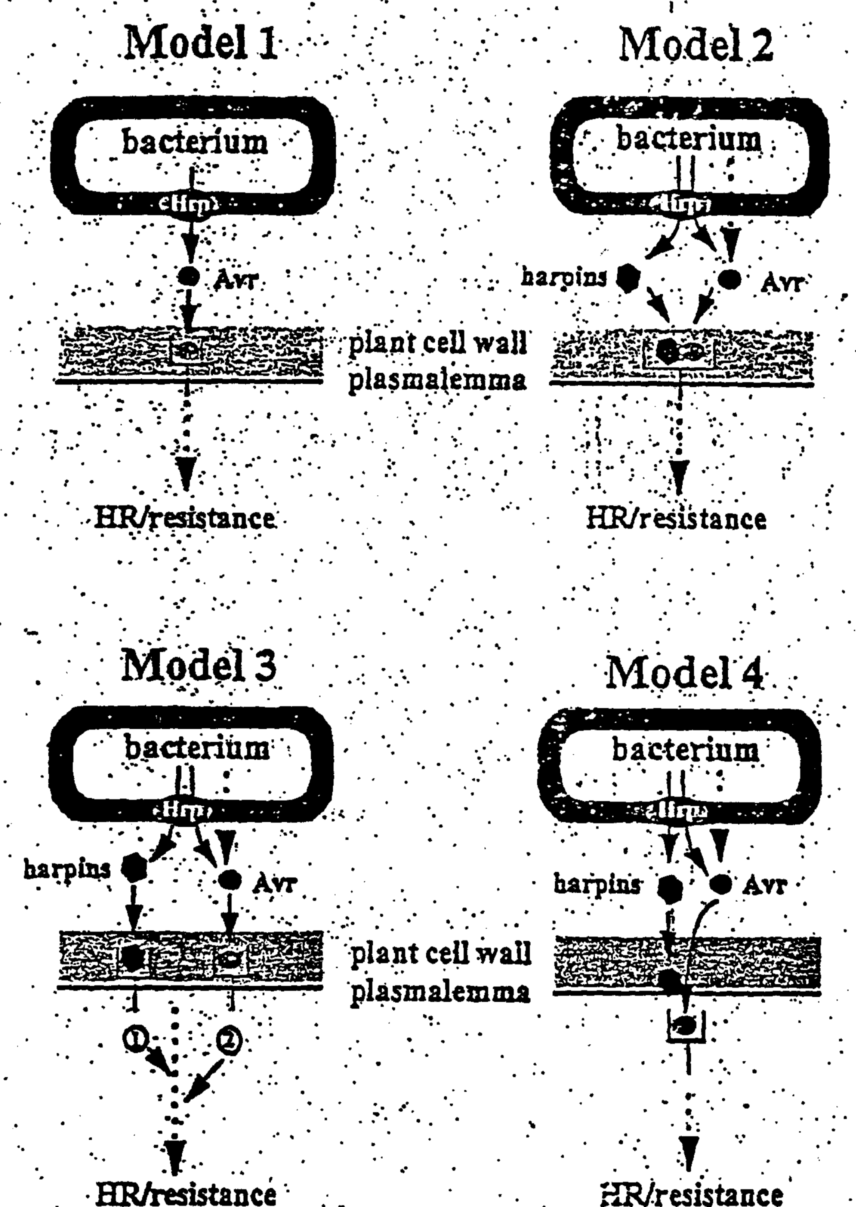


Fig. 4. Working models for possible interactions between *hrp* genes and *avr* genes. Model 1: Avr signals (Avr proteins or their enzymatic products) are secreted through the Hrp secretion apparatus to elicit the hypersensitive response (HR) and resistance. Model 2: Harpins and Avr signals modify each other before interacting with plant receptors. Avr signals may or may not be secreted via the Hrp secretion apparatus. Model 3: Harpins and Avr signals interact with respective plant receptors. Plant response elicited by harpins must precede plant response elicited by Avr signals. Avr signals may or may not be secreted via the Hrp secretion apparatus. Model 4: Avr proteins are secreted into the plant cell with the help of harpins. Avr signals may or may not be secreted via the Hrp secretion apparatus. In models 1 to 3, receptors for Avr proteins are presumed to be on the plant cell surface. In model 4, receptors for Avr proteins are inside the plant cell.

Acknowledgments

We thank Karen Bird for help in preparation of this paper, Paul Vincelli and Robert P. Scheffer for critical review, and Marlene Cameron for illustrations. Research in our laboratory is supported by grants from USDA and DOE.

Literature Cited

1. Agrios, G. N. 1988. Plant Pathology. 3rd ed. Academic Press, San Diego, CA.
2. Arlat, M., van Gijsegem, F., Huet, J. C., Pernollet, J. C., and Boucher, C. A. 1994. PopA1, a protein which induces a hypersensitivity-like response on specific Petunia genotypes, is secreted via the Hrp pathway of *Pseudomonas solanacearum*. EMBO J. 13:543-553.
3. Barta, T. M., Kinscherf, T. G., and Willis, D. K. 1992. Regulation of tabtoxin production by the *lemA* gene in *Pseudomonas syringae*. J. Bacteriol. 174:3021-3029.
4. Bauer, D. W., Wei, Z.-M., Beer, S. V., and Collmer, A. 1995. *Erwinia chrysanthemi* harpin$_{Ech}$: An elicitor of the hypersensitive response that contributes to soft-rot pathogenesis. Mol. Plant-Microbe Interact. 8:484-491.
5. Beer, S. V., Bauer, D. W., Jiang, X. H., Laby, R. J., Sneath, B. J., Wei, Z., Wilcox, D. A., and Zumoff, C. H. 1990. The *hrp* gene cluster of *Erwinia amylovora*. Pages 53-60 in: Advances in Molecular Genetics of Plant-Mi-

crobe Interactions, Vol. I. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht.
6. Bonas, U. 1994. *hrp* genes of phytopathogenic bacteria. Curr. Top. Microbiol. Immunol. 192:79-98.
7. Collmer, A., and Keen, N. T. 1986. The role of pectic enzymes in plant pathogenesis. Annu. Rev. Phytopathol. 24:383-409.
8. Dangl, J. L. 1995. Genes involved in bacterial pathogenesis of plants. Pages 293-304 in: Pathogenesis and Host Specificity in Plant Diseases: Histochemical, Biochemical, Genetic and Molecular Bases. Vol. I, Prokaryotes. U. S. Singh, R. P. Singh, and K. Kohmoto, eds. Pergamon, U.K.
9. Denny, T. P. 1995. Involvement of bacterial polysaccharide in plant pathogenesis. Annu. Rev. Phytopathol. 33:173-179.
10. Dixon, R. A., Harrison, M. J., and Lamb, C. J. 1994. Early events in the activation of plant defense responses. Annu. Rev. Phytopathol. 32:479-501.
11. El-Banoby, F. E., and Rudolph, K. 1979. Induction of water-soaking in plant leaves by extracellular polysaccharides from phytopathogenic pseudomonads and xanthomonads. Physiol. Plant Pathol. 15:341-349.
12. Fenselau, S., Balbo, I., and Bonas, U. 1992. Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. Mol. Plant-Microbe Interact. 5:390-396.
13. Fett, W. F., and Dunn, M. F. 1989. Exopolysaccharides produced by phytopathogenic *Pseudomonas syringae* pathovars in infected leaves of susceptible hosts. Plant Physiol. 89:5-9.
14. Flor, A. H. 1947. Host-parasite interactions in flax rust - its genetics and other implications. Phytopathology 45:680-685.
15. Genin, S., Gough, C. L., Zischek, C., and Boucher, C. A. 1992. Evidence that the *hrpB* gene encodes a positive regulator of pathogenicity genes from *Pseudomonas solanacearum*. Mol. Microbiol. 6:3065-3076.
16. Goodman, R. N., and Novacky, A. 1994. The hypersensitive reaction in plants to pathogens: A resistance phenomenon. American Phytopathological Society, St. Paul, MN.
17. Gough, C. L., Genin, S., Zischek, C., and Boucher, C. A. 1992. *hrp* genes of *Pseudomonas solanacearum* are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. Mol. Plant-Microbe Interact. 5:384-389.
18. Grimm, C., and Panopoulos, N. J. 1989. The predicted protein product of a pathogenicity locus from *Pseudomonas syringae* pv. *phaseolicola* is homologous to a highly conserved domain of several procaryotic regulatory proteins. J. Bacteriol. 171:5031-5038.
19. Gross, D. C. 1991. Molecular and genetic analysis of toxin production by pathovars of *Pseudomonas syringae*. Annu. Rev. Phytopathol. 29:247-278.
20. He, S. Y., Huang, H.-C., and Collmer, A. 1993. *Pseudomonas syringae* pv. *syringae* harpin$_{Pss}$: A protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. Cell 73:1255-1266.
21. He, S. Y., Lindeberg, M. L., and Collmer, A. 1993. Protein secretion by plant pathogenic bacteria. Pages 39-64 in: Biotechnological Prospects for Plant Pathogen Control. I. Chet, ed. John Wiley & Sons, New York.
22. Huang, H.-C., He, S. Y., Bauer, D. W., and Collmer, A. 1992. The *Pseudomonas syringae* pv. *syringae* 61 *hrpH* product: An envelope protein required for elicitation of the hypersensitive response in plants. J. Bacteriol. 174:6878-6885.
23. Huang, H.-C., Lin, R.-H., Chang, C.-J., Collmer, A., and Deng, W.-L. 1995. The complete *hrp* gene cluster of *Pseudomonas syringae* pv. *syringae* 61 includes two blocks of genes required for harpin$_{Pss}$ secretion that are arranged colinearly with *Yersinia ysc* homologs. Mol. Plant-Microbe Interact. 8:733-746.
24. Huang, H. C., Schuurink, R., Denny, T. P., Atkinson, M. M., Baker, C. J., Yucel, I., Hutcheson, S. W., and Collmer, A. 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco. J. Bacteriol. 170:4748-4756.
25. Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: Regulation of a pathogen gene determining host cultivar specificity. Science 245:1374-1377.
26. Innes, R. W., Bent, A. F., Kunkel, B. N., Bisgrove, S. R., and Staskawicz, B. J. 1993. Molecular analysis of avirulence gene *avrRpt2* and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes. J. Bacteriol. 175:4859-4869.
27. Keen, N. T. 1990. Gene-for-gene complementarity in plant-pathogen interactions. Annu. Rev. Genet. 24:447-463.
28. Keen, N. T., Tamaki, S., Kobayashi, D., Gerhold, D., Stayton, M., Shen, H., Gold, S., Lorang, J., Thordal-Christensen, H., Dahlbeck, D., and Staskawicz, B. 1990. Bacteria expressing avirulence gene D produce a specific elicitor of the soybean hypersensitive reaction. Mol. Plant-Microbe Interact. 3:112-121.
29. Klement, Z. 1963. Rapid detection of pathogenicity of phytopathogenic pseudomonads. Nature 199:299-300.
30. Knoop, V., Staskawicz, B., and Bonas, U. 1991. Expression of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria* is not under the control of *hrp* genes and is independent of plant factors. J. Bacteriol. 173:7142-7150.
31. Laby, R. J., and Beer, S. V. 1992. Hybridiza

Suresh Gopalan

Dr. Gopalan is a research associate at the Department of Energy Plant Research Laboratory, Michigan State University. He received his B.S. degree in mechanical engineering and his M.S. degree in bioscience under the dual degree program at the Birla Institute of Technology and Science, India, in 1983. He received his Ph.D. degree in biotechnology at the Center for Biotechnology, Anna University, India, in 1994. His thesis was on the development of a biopesticide based on *Bacillus sphaericus* for mosquito control. He joined S. Y. He's laboratory in October 1993 as a postdoctoral fellow at the University of Kentucky. In 1995, he moved with He to th DOE Plant Research Laboratory His current research focuses on molecular aspects of plant responses to bacterial pathogens.

S. Y. He

Dr. He is an assistant professor in the Department of Energy Plant Research Laboratory at Michigan State University. He received his B.S. and M.S. degrees in plant protection at Zhejiang Agricultural University, People's Republic of China, and his Ph.D. degree in plant pathology at Cornell University. He joined the faculty of the University of Kentucky in 1993. In 1995, he moved to Michigan State University. His research interests are in molecular plant-microbe interactions.

cation and functional complementation of the *hrp* gene cluster from *Erwinia amylovora* strain Ea321 with DNA of other bacteria. Mol. Plant-Microbe Interact. 5:412-419.

32. Lamb, C. J., Lawton, M. A., Dron, M., and Dixon, R. A. 1989. Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell 56:215-224.
33. Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. 1986. Gene cluster of *Pseudomonas syringae* pv. *phaseolicola* controls pathogenicity of bean plants and hypersensitivity on nonhost plants. J. Bacteriol. 168:512-522.
34. Martin, G. B., Brommonschenkel, S. H., Chunwongse, J., Frary, A., Ganal, M. W., Spivey, R., Wu, T., Earle, E. D., and Tanksley, S. D. 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262:1432-1436.
35. Niepold, F., Anderson, D., and Mills, D. 1985. Cloning determinants of pathogenesis from *Pseudomonas syringae* pathovar *syringae*. Proc. Natl. Acad. Sci. USA 82:406-410.
36. Preston, G., Huang, H.-C., He, S. Y., and Collmer, A. 1995. The HrpZ proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea*, and *tomato* are encoded by an operon containing *Yersinia ysc* homologs and elicit the hypersensitive response in tomato but not in soybean. Mol. Plant-Microbe Interact. 8:717-732.
37. Rahme, L. G., Mindrinos, M. N., and Panopoulos, N. J. 1992. Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syringae* pv. *phaseolicola*. J. Bacteriol. 174:3499-3507.
38. Salmeron, J. M., and Staskawicz, B. J. 1993. Molecular characterization and *hrp* dependence of the avirulence gene *avrPto* from *Pseudomonas syringae* pv. *tomato*. Mol. Gen. Genet. 239:6-16.
39. Salmond, G. C. 1994. Secretion of extracellular virulence factors by plant pathogenic bacteria. Annu. Rev. Phytopathol. 32:181-200.
40. Shen, H., and Keen, N. T. 1993. Characterization of the promoter of avirulence gene D from *Pseudomonas syringae* pv. *tomato*. J. Bacteriol. 175:5916-5924.
41. Shulte, R., and Bonas, U. 1992. A *Xanthomonas* pathogenicity locus is induced by sucrose and sulfur-containing amino acids. Plant Cell 4:79-86.
42. Silverstone, S. E., Gilchrist, D. G., Bostock, R. M., and Kosuge, T. 1993. The 73-kb pIAA plasmid increases competitive fitness of *Pseudomonas syringae* subspecies *savastanoi* in oleander. Can. J. Plant Sci. Biotechnol. Agric. 14:659-664.
43. Staskawicz, B. J., Ausubel, F. M., Baker, B. J., Ellis, J. G., and Jones, J. D. G. 1995. Molecular genetics of plant disease resistance. Science 268:661-667.
44. Staskawicz, B. J., Dahlbeck, D., and Keen, N. T. 1984. Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinea* determines race-specific incompatibility on *Glycine max* (L.) Merr. Proc. Natl. Acad. Sci. USA 81:6024-6028.
45. Van Gijsegem, F., Genin, S., and Boucher, C. 1995. *hrp* and *avr* genes, key determinants controlling the interactions between plants and Gram-negative phytopathogenic bacteria. Pages 273-292 in: Pathogenesis and Host Specificity in Plant Diseases: Histochemical, Biochemical, Genetic and Molecular Bases. Vol. I, Prokaryotes. U. S. Singh, R. P. Singh, and K. Kohmoto, eds. Pergamon, U.K.
46. Van Gijsegem, F., Gough, C., Zischek, C., Niqueux, E., Arlat, M., Genin, S., Barberis, P., German, S., Castello, P., and Boucher, C. 1995. The *hrp* gene locus of *Pseudomonas solanacearum*, which controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex. Mol. Microbiol. 15:1095-1114.
47. Wei, Z.-M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A., and Beer, S. V. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science 257:85-88.
48. Wei, Z.-M., Sneath, B. J., and Beer, S. V. 1992. Expression of *Erwinia amylovora hrp* genes in response to environmental stimuli. J. Bacteriol. 174:1875-1882.
49. Willis, D. K., Barta, T. M., and Kinscherf, T. G. 1991. Genetics of toxin production and resistance in phytopathogenic bacteria. Experientia 47:765-771.
50. Willis, D. K., Rich, J. J., and Hrabak, E. M. 1991. *hrp* genes of phytopathogenic bacteria. Mol. Plant-Microbe Interact. 4:132-138.
51. Xiao, Y., Heu, S., Yi, J., Lu, Y., and Hutcheson, S. W. 1994. Identification of a putative alternative sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes. J. Bacteriol. 176:1025-1036.
52. Xiao, Y., Lu, Y., Heu, S., and Hutcheson, S. W. 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 *hrp* cluster. J. Bacteriol. 174:1734-1741.
53. Yucel, I., Xiao, Y., and Hutcheson, S. V. 1989. Influence of *Pseudomonas syringae* culture conditions on initiation of the hypersensitive response of cultured tobacco cells. Appl. Environ. Microbiol. 55:1724-1729.

MPMI Vol. 8, No. 4, 1995, pp. 484–. 1995 The American Phytopathological Society

Erwinia chrysanthemi Harpin$_{Ech}$: An Elicitor of the Hypersensitive Response that Contributes to Soft-Rot Pathogenesis

David W. Bauer, Zhong-Min Wei, Steven V. Beer, and Alan Collmer

Department of Plant Pathology, Cornell University, Ithaca, NY 14853-4203 U.S.A.
Received 14 December 1994. Accepted 8 March 1995.

Mutants of the soft-rot pathogen *Erwinia chrysanthemi* EC16 that are deficient in the production of the pectate lyase isozymes PelABCE can elicit the hypersensitive response (HR) in tobacco leaves. The *hrpN$_{Ech}$* gene was identified in a collection of cosmids carrying *E. chrysanthemi hrp* genes by its hybridization with the *Erwinia amylovora hrpN$_{Ea}$* gene. *hrpN$_{Ech}$* appears to be in a monocistronic operon, and it encodes a predicted protein of 340 amino acids that is glycine-rich, lacking in cysteine, and highly similar to HrpN$_{Ea}$ in its C-terminal half. *Escherichia coli* DH5α cells expressing *hrpN$_{Ech}$* from the *lac* promoter of pBluescript II accumulated HrpN$_{Ech}$ in inclusion bodies. The protein was readily purified from cell lysates carrying these inclusion bodies by solubilization in 4.5 M guanidine-HCl and reprecipitation upon dialysis against dilute buffer. HrpN$_{Ech}$ suspensions elicited a typical HR in tobacco leaves, and elicitor activity was heat-stable. Tn*5-gusA1* mutations were introduced into the cloned *hrpN$_{Ech}$* and then marker-exchanged into the genomes of *E. chrysanthemi* strains AC4150 (wild type), CUCPB5006 (Δ*pelABCE*), and CUCPB5030 (Δ*pelABCE outD*::Tn*phoA*). *hrpN$_{Ech}$*::Tn*5-gusA1* mutations in CUCPB5006 abolished the ability of the bacterium to elicit the HR in tobacco leaves unless complemented with an *hrpN$_{Ech}$* subclone. An *hrpN$_{Ech}$*::Tn*5-gusA1* mutation also reduced the ability of AC4150 to incite infections in witloof chicory leaves, but it did not reduce the size of lesions that did develop. Purified HrpN$_{Ech}$ and *E. chrysanthemi* strains CUCPB5006 and CUCPB5030 elicited HR-like necrosis in leaves of tomato, pepper, African violet, petunia, and pelargonium, whereas *hrpN$_{Ech}$* mutants did not. HrpN$_{Ech}$ thus appears to be the only HR elicitor produced by *E. chrysanthemi* EC16, and it contributes to the pathogenicity of the bacterium in witloof chicory.

The hypersensitive response (HR) is a rapid, localized necrosis that is associated with the active defense of plants against many pathogens (Kiraly 1980; Klement 1982). The HR elicited by bacteria is readily observed as a tissue collapse if high concentrations (≥10^7 cells per milliliter) of a limited-host-range pathogen like *Pseudomonas syringae* or *Erwinia amylovora* are infiltrated into leaves of nonhost plants (necrosis occurs only in isolated plant cells at lower levels of inoculum) (Klement 1963; Klement *et al.* 1964; Turner and Novacky 1974; Klement 1982). The capacities to elicit the HR in a nonhost and to be pathogenic in a host appear linked. As noted by Klement (1982), these pathogens also cause physiologically similar, albeit delayed, necroses in their interactions with compatible hosts. Furthermore, the ability to produce the HR or pathogenesis is dependent on a common set of genes, denoted *hrp* (Lindgren *et al.* 1986; Willis *et al.* 1991). Consequently, the HR may hold clues to both the nature of plant defense and the basis for bacterial pathogenicity.

The *hrp* genes are widespread in gram-negative plant pathogens, where they are clustered, conserved, and in some cases interchangeable (Willis *et al.* 1991; Bonas 1994). Several *hrp* genes encode components of a protein secretion pathway similar to one used by *Yersinia, Shigella,* and *Salmonella* spp. to secrete proteins essential in animal diseases (Van Gijsegem *et al.* 1993). In *E. amylovora, P. syringae,* and *P. solanacearum, hrp* genes have been shown to control the production and secretion of glycine-rich protein elicitors of the HR (He *et al.* 1993; Wei and Beer 1993; Arlat *et al.* 1994).

The first of these proteins was discovered in *E. amylovora* Ea321, a bacterium that causes fire blight of rosaceous plants, and was designated harpin (Wei *et al.* 1992). Mutations in the encoding *hrpN* gene revealed that harpin is required for *E. amylovora* to elicit the HR in nonhost tobacco leaves and incite disease symptoms in highly susceptible pear fruit. The *P. solanacearum* GMI1000 PopA1 protein has similar physical properties and also elicits the HR in leaves of tobacco, which is not a host of that strain (Arlat *et al.* 1994). However, *P. solanacearum popA* mutants still elicit the HR in tobacco and incite disease in tomato. Thus, the role of these glycine-rich HR elicitors can vary widely among gram-negative plant pathogens.

E. chrysanthemi is unlike the bacterial pathogens that typically elicit the HR because it has a wide host range, rapidly kills and macerates host tissues, and secretes several isozymes of the macerating enzyme pectate lyase (Pel) (Barras *et al.* 1994). Nevertheless, PelABCE$^-$ and Out$^-$ (pectic enzyme secretion pathway) mutants of *E. chrysanthemi* EC16 cause a typical HR (Bauer *et al.* 1994). Furthermore, elicitation of the HR by *E. chrysanthemi* is dependent on an *hrp* gene that is conserved in *E. amylovora* and *P. syringae* and functions in the secretion of the *E. amylovora* harpin (Wei and Beer 1993; Bauer *et al.* 1994). Mutation of this gene significantly reduces the ability of *E. chrysanthemi* to incite lesions in susceptible

Corresponding author: Alan Collmer; E-mail: arc2@cornell.edu

witloof chicory leaves. These observations suggest that *E. chrysanthemi* also produces a harpin. We report here the cloning, characterization, and mutagenesis of the *E. chrysanthemi* $hrpN_{Ech}$ gene and an investigation of the role of its product in plant interactions.

RESULTS

Molecular cloning of the *E. chrysanthemi* $hrpN_{Ech}$ gene.

We previously isolated 18 cosmids containing *E. chrysanthemi* DNA sequences hybridizing with the *E. amylovora hrp* cluster (Bauer *et al.* 1994). The pattern of restriction fragments released from these cosmids indicated they all contained overlapping inserts from the same region of the *E. chrysanthemi* genome (data not shown). The cosmids were probed in colony blots with a 1.3-kb *Hin*dIII fragment from pCPP1084,

```
   1  AATCAGGAAACGAAATTATGCAAATTACCATCAAAGCGCACATCGGCGGTGATTTGGGCG
                          M Q I T I K A H I G G D L G
  61  TCTCCGGTCTCGGCCTCGGTGCTCAGGGACTGAAAGGACTGAATTCCGCGGCTTCATCGC
      V S G L G L G A Q G L K G L N S A A S S
 121  TGGGTTCCAGCGTGGATAAACTGAGCAGCACCATCGATAAGTTGACCTCGGCGCTGACTT
      L G S S V D K L S S T I D K L T S A L T
 181  CGATGATGTTTGGCGGCGCGCTGGCGCAGGGGCTGGGCGCCAGCTCGAAGGGGCTGGGGA
      S M M F G G A L A Q G L G A S S K G L G
 241  TGAGCAATCAACTGGGCCAGTCTTTCGGCAATGGCGCGCAGGGTGCCAGCAACCTGCTAT
      M S N Q L G Q S F G N G A Q G A S N L L
 301  CCGTACCGAAATCCGGCGGCGCCATGCGTTGTCAAAAATGTTTGATAAAGCGCTGGACGATC
      S V P K S G G D A L S K M F D K A L D D
 361  TGCTGGGTCATGACACCGTGACCAAGCTGACTAACCAGAGCAACCAACTGGCTAATTCAA
      L L G H D T V T K L T N Q S N Q L A N S
                                        *----> 439::Tn5-gusA1
 421  TGCTCAACGCCAGCCAGATGACCCAGGGTAATATGAATGCGTTCGGCAGCGGTGTGAACA
      M L N A S Q M T Q G N M N A F G S G V N
 481  ACGCACTGTCGTCCATTCTCGGCAACGGTCTCGGCCAGTCCATGAGTGGCTTCTCTCAGC
      N A L S S I L G N G L G Q S M S G F S Q
      546::Tn5-gusA1 <-------*
 541  CTTCTCTGGGGGCAGGCGGCTTGCAGGGCCTGAGCGGCGCGGGTGCATTCAACCAGTTGG
      P S L G A G G L Q G L S G A G A F N Q L
 601  GTAATGCCATCGGCATGGGCGTGGGGCAGAATGCTGCGCTGAGTGCGTTGAGTAACGTCA
      G N A I G M G V G Q N A A L S A L S N V
 661  GCACCCACGTAGACGGTAACAACCGCCACTTTGTAGATAAAGAAGATCGCGGCATGGCGA
      S T H V D G N N R H F V D K E D R G M A
 721  AAGAGATCGGCCAGTTTATGGATCAGTATCCGGAAATATTCGGTAAACCGGAATACCAGA
      K E I G Q F M D Q Y P E I F G K P E Y Q
 781  AAGATGGCTGGAGTTCGCCGAAGACGGACGACAAATCCTGGGCTAAAGCGCTGAGTAAAC
      K D G W S S P K T D D K S W A K A L S K
 841  CGGATGATGACGGTATGACCGGCGCCAGCATGGACAAATTCCGTCAGGCGATGGGTATGA
      P D D D G M T G A S M D K F R Q A M G M
 901  TCAAAAGCGCGGTGGCGGGTGATACCGGCAATACCAACCTGAACCTGCGTGGCGCGGGCG
      I K S A V A G D T G N T N L N L R G A G
 961  GTGCATCGCTGGGTATCGATGCGGCTGTCGTCGGCGATAAAATAGCCAACATGTCGCTGG
      G A S L G I D A A V V G D K I A N M S L
1021  GTAAGCTGGCCAACGCCTGATAATCTGTGCTGGCCTGATAAAGCCGAAACGAAAAAAGAG
      G K L A N A * *
1081  ACGGGGAAGCCTGTCTCTTTTCTTATTATGCGC    1113
```

Fig. 1. DNA sequence of $hrpN_{Ech}$ and predicted amino acid sequence of its product. Underlined are the putative ribosome-binding site, the N-terminal amino acids confirmed by sequencing the product of pCPP2172, and a potential rho-independent transcription terminator. The location and orientation of two Tn*5-gusA1* insertions are also indicated and are numbered according to their location in the $hrpN_{Ech}$ open reading frame. The accession number for *hrpN* is L39897.

which contains the *E. amylovora hrpN* gene (Wei *et al.* 1992). pCPP2157, one of the three cosmids hybridizing with the probe, was digested with several restriction enzymes, and the location of the $hrpN_{Ech}$ gene in those fragments was determined by probing a Southern blot with the *E. amylovora Hin*dIII fragment. Two fragments, each containing the entire $hrpN_{Ech}$ gene, were subcloned into different vectors: pCPP2142 contained an 8.3-kb *Sal*I fragment in pUC119 (Vieira and Messing 1987), and pCPP2141 contained a 3.1-kb *Pst*I fragment in pBluescript II SK(–) (Stratagene, La J lla, CA).

Sequence of $hrpN_{Ech}$.

The nucleotide sequence of a 2.4-kb region of pCPP2141 encompassing $hrpN_{Ech}$ was determined. The portion of that sequence extending from the putative ribosome-binding site through the $hrpN_{Ech}$ coding sequence to a putative rho-independent terminator is presented in Figure 1. The typical ribosome-binding site, consisting of GGAAA, was located eight bases upstream of the ATG translational initiation codon. A consensus *hrp* promoter sequence of $GGAACC(N)_{16}CACTCA$ (Bonas 1994) was found 97 bases upstream of the open reading frame (ORF), suggesting that $hrpN_{Ech}$ is a monocistronic operon. $hrpN_{Ech}$ codes for a predicted protein that has a molecular mass of 34.3 kDa, is rich in glycine (16.2%), and is lacking in cysteine. Comparison of the amino acid sequences of the predicted $hrpN_{Ea}$ and $hrpN_{Ech}$ products revealed extensive similarity, particularly in the C-terminal halves of the proteins (Fig. 2). The overall identity of the

```
Ech .......MQITIKAHIGGDLGVSGLGLGAQGLKGLNSAASSLGSSVDKL 42
Ea  MSLNTSGLGASTMQISIGGAGGNNGL.LGTSRQNAGLGGNSALGLGGGNQ 49

Ech SSTIDKLTSALTSMMF.......GGALAQGLGAS.SKGLGMSNQLGQSFG 84
Ea  NDTVNQLAGLLTGMMMMMSMMGGGGLMGGGLGGGLGNGLGGSGGLGEGLS 99

Ech NGAQG..ASNLLSVPKSGGDALSKMFDKALDDLLG............... 117
Ea  NALNDMLGGSLNTLGSKGGNNTTSTTNSPLDQALGINSTSQNDDSTSGTD 149

Ech .....HDTVTKLTNQSNQLANSMLNAS............QMTQGNMNAFG 150
Ea  STSDSSDPMQQLLKMFSEIMQSLFGDGQDGTQGSSSGGKQPTEGEQNAYK 199

Ech SGVNNALSSILGNGLGQSMS...............GFSQPSLGAGGLQGLS 186
Ea  KGVTDALSGLMGNGLSQLLGNGGLGGGQGGNAGTGLDGSSLGGKGLRGLS 249

Ech GAGAFNQLGNAIGMGVGQNAALSALSNVSTHVDGNNRHFVDKEDRGMAKE 236
Ea  GPVDYQQLGNAVGTGIGMKAGIQALNDIGTHRHSSTRSFVNKGDRAMAKE 299

Ech IGQFMDQYPEIFGKPEYQKDGWSSPKTDDKSWAKALSKPDDDGMTGASMD 286
Ea  IGQFMDQYPEVFGKPQYQKGPGQEVKTDDKSWAKALSKPDDDGMTPASME 349

Ech KFRQAMGMIKSAVAGDTGNTNLNLRGAGGASLGIDAAVVGDKIANMSLGK 336
Ea  QFNKAKGMIKRPMAGDTGNGNLH...........DAVPVVLRWVLMP... 385

Ech LANA 340
```

Fig. 2. Predicted amino acid sequences f the *hrpN* products $HrpN_{Ech}$ (Ech) f *Erwinia chrysanthemi* and $HrpN_{Ea}$ (Ea) of *E. amylovora*, aligned by the Gap program of the Genetics Computer Group Sequence Analysis Software Package (Devereaux *et al.* 1984). Two dots denote greater similarity than one dot.

hrpN genes and proteins was 66.9 and 45.5%, as determined by the FASTA and Gap algorithms, respectively (Devereaux *et al.* 1984; Pearson and Lipman 1988).

The direction of *hrpN*$_{Ech}$ transcription, the size of the predicted product, and the translation start site were confirmed by recloning the 3.1-kb *Pst*I fragment from pCPP2157 and selecting a clone with the fragment in pBluescript II SK(–) in the opposite orientation from pCPP2141, to produce pCPP2172. *E. coli* DH5α(pCPP2172) expressed *hrpN*$_{Ech}$ from the vector *lac* promoter and produced high levels of a protein with an estimated molecular mass of 36 kDa in sodium dodecyl sulfate (SDS) polyacrylamide gels, which is close to the predicted size (Fig. 3). Furthermore, the 10 N-terminal amino acids of the 36-kDa protein, determined by microsequencing following purification as described below, corresponded with the predicted N terminus of HrpN$_{Ech}$. As expected, no N-terminal signal sequence for targeting to the general export (Sec) pathway was discernible in the HrpN$_{Ech}$ sequence, and our data showed no evidence of processing of the N terminus.

Purification of the *hrpN*$_{Ech}$ product and demonstration of its HR elicitor activity in tobacco.

When DH5α(pCPP2172) cells were disrupted by sonication and then centrifuged, most of the HrpN$_{Ech}$ protein sedimented with the cell debris. However, soluble HrpN$_{Ech}$ could be released from this material by treatment with 4.5 M guanidine-HCl. This suggested that the protein formed inclusion bodies which could be exploited for purification. As detailed in Materials and Methods, we found that HrpN$_{Ech}$ reprecipitated when the guanidine-HCl was removed by dialysis against dilute buffer. The HrpN$_{Ech}$ precipitate could be washed and resuspended in buffer, in which it formed a fine suspension. SDS polyacrylamide gel analysis revealed the suspension to be electrophoretically homogeneous HrpN$_{Ech}$ (Fig. 3).

Cell-free lysates from *E. coli* DH5α(pCPP2172) cells grown in Luria-Bertani medium were infiltrated into tobacco leaves. Necrosis typical of the HR developed within 18 h, whereas leaf panels infiltrated with identically prepared lysates of DH5α(pBluescript SK–) showed no response (data not shown). The suspension of purified HrpN$_{Ech}$ at a concentration of 336 μg/ml also caused a necrotic response within 18 h that was indistinguishable from that caused by *E. chrysanthemi* CUCBP5030 or cell-free lysates from *E. coli* DH5α(pCPP2172) (Fig. 4). Tobacco plants vary in their sensitivity to harpins, and elicitation of the HR by HrpN$_{Ech}$ at lower concentrations was found to be variable. Consequently, a concentration of 336 μg/ml was used in all subsequent experiments. The concentration of HrpN$_{Ech}$ that is soluble in apoplastic fluids is unknown. To determine the heat stability of HrpN$_{Ech}$, the suspension of purified protein was incubated at 100° C for 15 min and then infiltrated into a tobacco leaf. There was no apparent diminution in its ability to elicit the HR (data not shown). These observations indicated that HrpN$_{Ech}$ is sufficient to account for the ability of *E. chrysanthemi* to elicit the HR in tobacco.

***hrpN*$_{Ech}$ mutants fail to elicit the HR in tobacco.**

E. coli DH10B(pCPP2142) was mutagenized with Tn*5-gusA1* (Sharma and Signer 1990). Plasmid DNA was isolated

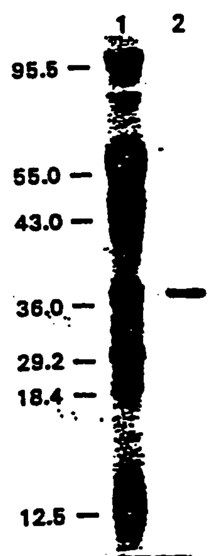


Fig. 3. Sodium dodecyl sulfate (SDS) polyacrylamide gel of purified HrpN$_{Ech}$. Purified HrpN$_{Ech}$ was solubilized in SDS loading buffer, electrophoresed through a 12% polyacrylamide gel, and stained with Coomassie Brilliant Blue. Lane 1, molecular weight markers (mid-range markers from Diversified Biotech, Boston, MA), with sizes in kilodaltons shown to the left; lane 2, HrpN$_{Ech}$.

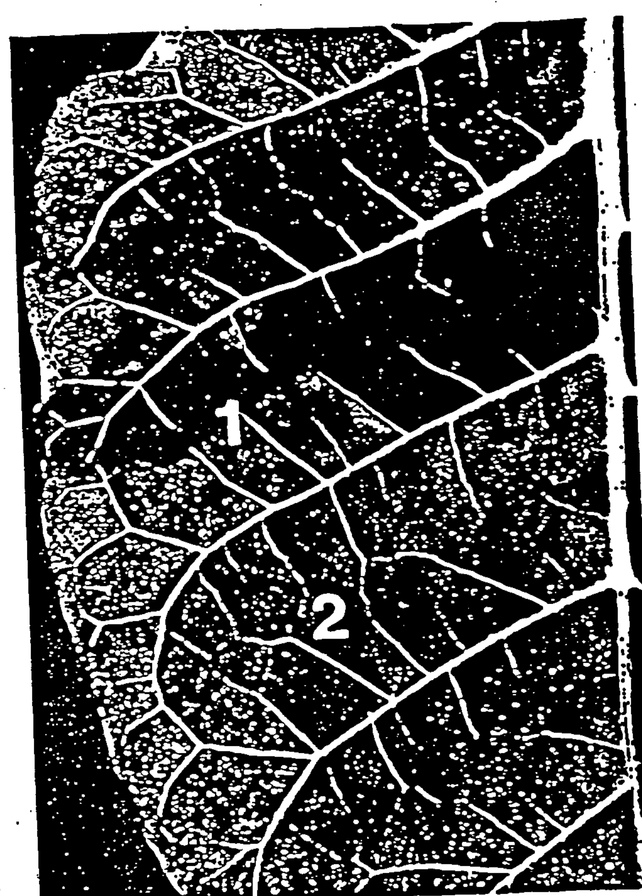


Fig. 4. Response f tobacco leaf tissue to purified HrpN$_{Ech}$. Leaf panel 1 was infiltrated with a suspension f purified HrpN$_{Ech}$ at a concentration of 336 μg/ml in 5 mM morpholinoethanesulfonic acid, pH 6.5. Panel 2 was infiltrated with buffer alone. The tissue in panel 1 collapsed 18 hr later. The leaf was photographed, 24 hr after infiltration, with a cross-polarized transilluminator, which enhances black and white visualization by making necrotic, desiccated areas that are typical of the hypersensitive response appear black.

from kanamycin-resistant colonies and transformed into *E. coli* DH5α, with selection for kanamycin resistance. Plasmids containing Tn*5-gusA1* were analyzed by restriction mapping. Two independent insertions in an 0.82-kb *Cla*I fragment internal to $hrpN_{Ech}$ were chosen for further study. The precise location and orientation f these insertions was determined by using a primer that hybridizes to Tn*5-gusA1* DNA upstream of *gusA* to sequence into the disrupted *E. chrysanthemi* DNA (Fig. 1). *E. coli* DH5α(pCPP2142) cells carrying the Tn*5-gusA1* insertion at nucleotide 439 of the $hrpN_{Ech}$ ORF (with *gusA* and $hrpN_{Ech}$ in the same orientation) produced dark blue colonies indicative of β-glucuronidase activity on LM agar (Hanahan 1983) supplemented with 5-bromo-4-chloro-3-indolyl β-D-glucuronide (data not shown). Whether *gusA* was expressed from an *E. chrysanthemi* promoter or the vector *lac* promoter was not determined. The $hrpN_{Ech}439$::Tn*5-gusA1* and $hrpN_{Ech}546$::Tn*5-gusA1* mutations were marker-exchanged into the genome of *E. chrysanthemi* CUCPB5006 (Δ*pelABCE*) to produce mutants CUCPB5046 and CUCPB-5045, respectively. Neither of the $hrpN_{Ech}$ mutants elicited a visible reaction in tobacco leaves (Fig. 5).

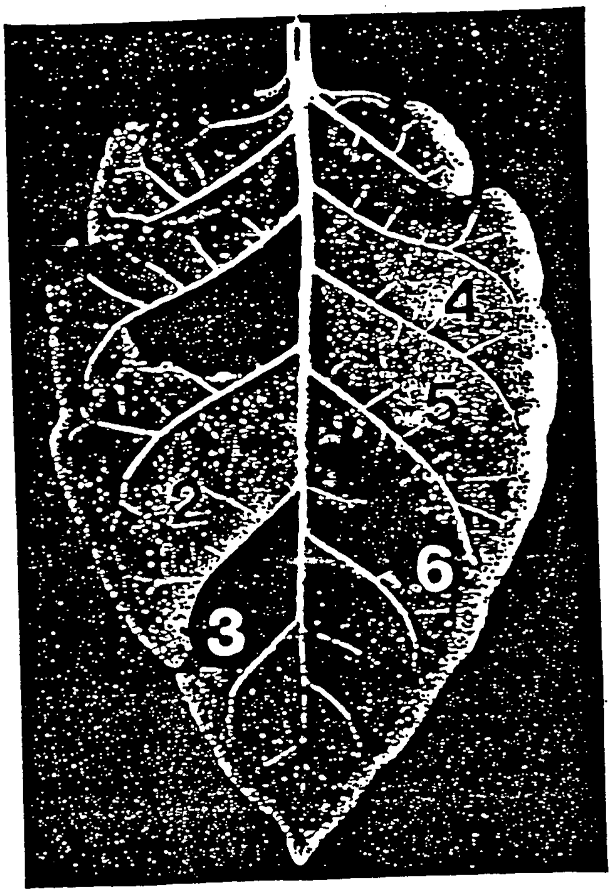


Fig. 5. Tobacco leaf showing that *Erwinia chrysanthemi hrpN* mutants do not elicit the hypersensitive response unless complemented with *hrpN*⁺ pCPP2174. Bacteria were suspended at a concentration of 5×10^8 cells per milliliter in 5 mM morpholinoethanesulfonic acid, pH 6.5, and infiltrated into a tobacco leaf. The leaf was photographed 24 hr later under cross-polarized transillumination, as in Figure 4. 1, *E. chrysanthemi* CUCPB5006 (Δ*pelABCE*); 2, CUCPB5045 (Δ*pelABCE* $hrpN_{Ech}546$::Tn*5-gusA1* derivative of CUCPB5006); 3, CUCPB5045(pCPP2174); 4, buffer alone; 5, CUCPB5046 (Δ*pelABCE* $hrpN_{Ech}439$::Tn*5-gusA1* derivative of CUCPB5006); 6, CUCPB5046(pCPP2174).

E. chrysanthemi $hrpN_{Ech}$ mutations can be complemented *in trans* with $hrpN_{Ech}$ but not with $hrpN_{Ea}$.

The presence of a typical rho-independent terminator just downstream of the $hrpN_{Ech}$ ORF suggested that mutations in the gene would not have polar effects on any other genes and that the HR elicitation phenotype should be restored by an $hrpN_{Ech}$ subclone. Because pCPP2172 carried 2 kb f *E. chrysanthemi* DNA in addition to $hrpN_{Ech}$, we constructed a precise subclone of the gene for this purpose. Oligonucleotides were used to amplify the $hrpN_{Ech}$ ORF by polymerase chain reaction and to introduce terminal *Nco*I and *Xho*I sites. The introduction of the restriction sites resulted in changing the second residue in the protein from glutamine to valine and adding a leucine and a glutamic acid residue to the C terminus. The resulting DNA fragment was ligated into *Xho*I- and *Nco*I-digested pSE280, creating pCPP2174, in which $hrpN_{Ech}$ was under control of the vector *tac* promoter. *E. chrysanthemi* CUCPB5045(pCPP2174) and CUCPB5046(pCPP2174) possessed HR elicitor activity (Fig. 5). HR elicitor activity could also be restored to these mutants by pCPP2142 and pCPP2172, but not by pCPP2141 (data not shown). Thus, the production of $HrpN_{Ech}$ is essential for elicitation of the HR by *E. chrysanthemi* CUCPB5006.

The feasibility of testing the interchangeability of the *hrpN* genes of *E. chrysanthemi* and *E. amylovora* was supported by the observation that HR elicitation activity could be restored to *hrpN* mutants in each species (*E. chrysanthemi* CUCPB-5045 and *E. amylovora* Ea321T5) by their respective *hrpN*⁺ subclones (pCPP2142 and pCPP1084). pCPP2142 was used for this purpose because preliminary immunoblot experiments indicated that the level of $hrpN_{Ech}$ expression by this plasmid, though relatively high, most closely approximated the expression of the native *hrpN* gene in *E. amylovora*. However, despite good heterologous expression of the *hrpN* genes, HR elicitation activity was not restored in either *E. amylovora* Ea321T5(pCPP2142) or *E. chrysanthemi*(pCPP1084) (data not shown). Thus, the genes do not appear to be functionally interchangeable.

E. chrysanthemi $hrpN_{Ech}$ mutants have a reduced ability to incite lesions in witloof chicory.

The $hrpN_{Ech}439$::Tn*5-gusA1* mutation was marker-exchanged into the genome of wild-type strain AC4150. The resulting mutant, CUCPB5049, was analyzed for its virulence in witloof chicory. Leaves were inoculated at small wounds with

Table 1. Effects of $hrpN_{Ech}$ mutation on the ability of *Erwinia chrysanthemi* to incite lesions on witloof chicory leaves

Strain	Number of lesions per 20 inoculations[a]	Size of lesions (mm^2, mean ± SD)[b]
AC4150 (wild type)	16	80 ± 55
CUCPB5049 ($hrpN_{Ech}429$::Tn*5-gusA1*)	8[c]	89 ± 42

[a] Each witloof chicory leaf was inoculated at two equivalent sites with 2×10^4 bacterial cells: one site received the $hrpN_{Ech}$ mutant, the other the parental wild-type strain; lesions were indicated by browning and maceration around the site of inoculation.
[b] Product of the length and width of the lesion.
[c] Different from the wild-type strain ($P < 0.05$), as determined by the McNemar test (Conover 1980).

2 x 10⁴ cells f mutant and wild-type strains, as previously described (Bauer *et al.* 1994). The level of inoculum corresponded with the experimentally determined ED_{50} of the wild-type strain for the batch of chicory heads used. The approximate surface area of macerated lesions was determined 72 h after inoculation. The mutations did not abolish the pathogenicity of *E. chrysanthemi*, but they significantly reduced the number of successful lesions (Table 1). However, the *hrpN*$_{Ech}$ mutation had no significant effect on the size of the lesions produced in successful infections.

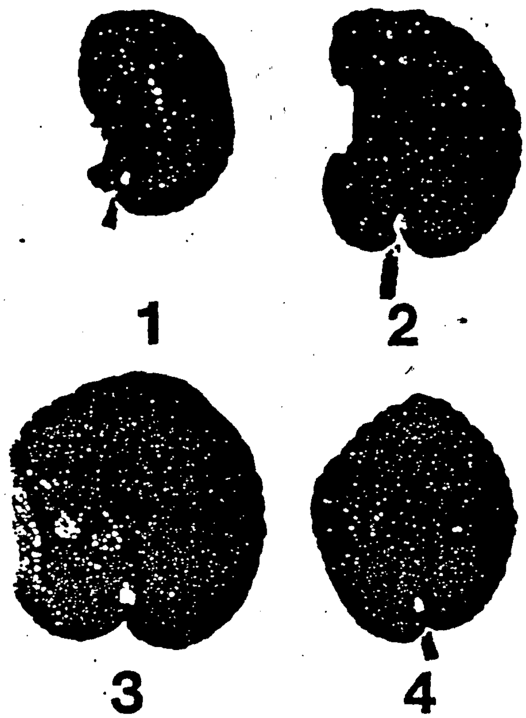


Fig. 6. African violet leaves showing rapid necrosis elicited by HrpN$_{Ech}$ and HrpN$^{+}_{Ech}$ Pel-deficient strains of *Erwinia chrysanthemi*. Leaves were inoculated with bacteria at a concentration of 3 × 10^8 cells per milliliter in 5 mM morpholinoethanesulfonic acid, pH 6.5, or purified HrpN$_{Ech}$ at a concentration of 336 μg/ml and photographed 24 hr later under cross-polarized transillumination, as in Figure 4. Buffer controls elicited no visible response (not shown). 1, *E. chrysanthemi* CUCPB5006 (Δ*pelABCE*); 2, CUCPB5030 (*outD*::Tn*phoA* derivative of CUCPB-5006); 3, HrpN$_{Ech}$; 4, (left) CUCPB5045 (Δ*pelABCE hrpN*$_{Ech}$*546*::Tn*5-gusA1* derivative of CUCPB5006) and (right) CUCPB5063 (Δ*pelABCE outD*::Tn*phoA hrpN*$_{Ech}$*546*::Tn*5-gusA1* derivative of CUCPB5006).

Elicitation of a rapid necrosis in several plants by *E. chrysanthemi* is dependent on HrpN$_{Ech}$.

To determine whether *E. chrysanthemi* could cause an HrpN$_{Ech}$-dependent necrosis in plants other than tobacco, a variety of plants were infiltrated with purified HrpN$_{Ech}$ or inoculated with Pel-deficient *E. chrysanthemi* strains. The strains used were CUCPB5006; its *hrpN*$_{Ech}$*546*::Tn*5-gusA1* derivative, CUCPB5045; CUCPB5030 (Δ*pelABCE outD*::Tn*phoA*); and its *hrpN*$_{Ech}$*546*::Tn*5-gusA1* derivative, CUCPB-5063. The results for African violet are shown in Figure 6, and results for all plants are summarized in Table 2. They yield several general observations. Plants responded either to both isolated HrpN$_{Ech}$ and *hrpN*$^{+}_{Ech}$ bacteria or to neither. Plants that responded to either treatment produced a non-macerated, HR-like necrosis that developed between 12 and 24 h after infiltration. *hrpN*$_{Ech}$ mutants failed to elicit a response in any of the plants. The *out* mutation had no apparent influence on the responses elicited in the plants tested, indicating that residual Pel isozymes or other proteins traveling the Out pathway were not involved in producing the HR-like necrosis. The results argue that HrpN$_{Ech}$ is the only elicitor of the HR produced by *E. chrysanthemi*.

DISCUSSION

E. chrysanthemi was found to produce a protein with many similarities to the harpin of *E. amylovora*. The two proteins share significant amino acid sequence identity, similar physical properties, and the ability to elicit the HR in a variety of plants. Mutations in the *hrpN*$_{Ech}$ gene indicate that, as with *E. amylovora*, harpin production is required for elicitation of the HR. Furthermore, both harpins contribute to bacterial pathogenicity, albeit to different degrees. HrpN$_{Ea}$ is essential for *E. amylovora* to produce symptoms in highly susceptible, immature pear fruit (Wei *et al.* 1992), whereas HrpN$_{Ech}$ merely increases the frequency of successful *E. chrysanthemi* infections in susceptible witloof chicory leaves. Nevertheless, the finding that harpins play some role in the pathogenicity f

Table 2. Elicitation of necrosis in various plants by HrpN$_{Ech}$ and by *Erwinia chrysanthemi* strains variously deficient in Pel production and HrpN$_{Ech}$ production

Plant	HrpN$_{Ech}$ [a]	CUCPB5006 (Δ*pelABCE*)[b]	CUCPB5045 (Δ*pelABCE hrpN*$_{Ech}$*546*:: Tn*5-gusA1*)	CUCPB5030 (Δ*pelABCE outD*::Tn*phoA*)	CUCPB5063 (Δ*pelABCE outD*::Tn*phoA hrpN*$_{Ech}$*546*:: Tn*5-gusA1*)
Tobacco	+	+	−	+	−
Tomato	+	+	−	+	−
Pepper	+	+	−	+	−
African violet	+	+	−	+	−
Petunia	+	+	−	+	−
Pelargonium	+	+	−	+	−
Squash	−	−	−	−	−
Zinnia	−	−	−	−	−

[a] Leaves on plants were infiltrated with HrpN$_{Ech}$ at a concentration of 336 μg/ml and observed macroscopically 24 hr later for necrosis and collapse of the infiltrated area (+) or absence of any response (−).
[b] Leaves on plants were infiltrated with bacteria at a concentration of 5 × 10^8/ml and scored for responses as described above.

such disparate pathogens suggests that these proteins have a conserved and widespread function in bacterial plant pathogenesis. We will consider below HrpN$_{Ech}$ with regard to the protein secretion pathways, extracellular virulence proteins, and wide host range of *E. chrysanthemi*.

E. chrysanthemi secretes proteins by multiple, independent pathways. Several protease isozymes are secreted by the Sec-independent (ABC-transporter, or Type I) pathway; pectic enzymes and cellulase are secreted by the Sec-dependent (general secretion, or Type II) pathway; and, HrpN$_{Ech}$ is likely to be secreted by the Sec-independent Hrp (Type III) pathway (Salmond 1994). The expectation that HrpN$_{Ech}$ is secreted by the Hrp pathway is supported by several lines of indirect evidence: (i) Hrp secretion pathway mutants have revealed that other members of this class of glycine-rich, heat-stable elicitor proteins—the *E. amylovora* HrpN$_{Ea}$, *P. syringae* pv. *syringae* HrpZ, and *P. solanacearum* PopA1 proteins—are secreted by this pathway (He *et al.* 1993; Wei and Beer 1993; Arlat *et al.* 1994); (ii) mutation of the *E. chrysanthemi* homolog of an *E. amylovora* gene involved in HrpN$_{Ea}$ secretion abolishes the ability of *E. chrysanthemi* to elicit the HR, whereas mutation of the Out (Type II) pathway of *E. chrysanthemi* does not abolish the HR; and (iii) HrpN$_{Ech}$ appears to be the only HR elicitor produced by *E. chrysanthemi* (as discussed further below), suggesting that the effect of the putative *hrp* secretion gene mutation is on HrpN$_{Ech}$. Our attempts to directly demonstrate *hrp*-dependent secretion of HrpN$_{Ech}$ have been thwarted by the apparent instability of the protein in *E. chrysanthemi*. Using the cell fractionation and immunoblotting procedures of He *et al.* (1993) and polyclonal anti-HrpN$_{Ea}$ antibodies that cross-react with HrpN$_{Ech}$ (Wei *et al.* 1992), we have observed the presence of HrpN$_{Ech}$ in the cell-bound fraction of *E. chrysanthemi* (D. W. Bauer, unpublished). However, some culture preparations unexpectedly lack the protein, and no preparations reveal accumulation of the protein in the culture supernatant fraction. It is possible that HrpN$_{Ech}$ aggregates upon secretion and therefore precipitates from the medium. It is interesting that several of the *Yersinia* spp. Yop virulence proteins aggregate in the medium upon secretion via the Type III pathway (Michiels *et al.* 1990). Similarly, HrpN$_{Ea}$ has a propensity to form aggregates or to associate with an insoluble membrane fraction (Wei *et al.* 1992).

It is significant that there is little difference in the plant interaction phenotypes of *E. chrysanthemi* mutants deficient in either HrpN$_{Ech}$ or a putative component of the Hrp secretion pathway (Bauer *et al.* 1994). Both mutations abolish the ability of Pel-deficient strains to elicit the HR, and they both reduce the frequency of successful infections incited by fully pectolytic strains in witloof chicory leaves without affecting

Table 3. Bacterial strains and plasmids used in this study

Designation	Relevant characteristic[a]	Reference or source
Escherichia coli		
ED8767	*supE44 supF58 hsdS3*($r_B^- m_B^-$) *recA56 galk2 galT22 metB1*	Sambrook *et al.* 1989
DH5α	*supE44* Δ*lac*U169 (φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* Nal^r	Hanahan 1983 Life Technologies, Inc., Grand Island, NY
DH10B	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80 *lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara, leu*)*7697 galU galK rpsL nupG*	Grant *et al.* 1990 Life Technologies, Inc.
Erwinia chrysanthemi		
EC16	Wild-type strain	Burkholder *et al.* 1953
AC4150	Spontaneous Nal^r derivative of EC16	Chatterjee *et al.* 1983
CUCPB5006	Δ(*pelB pelC*)::28bp Δ(*pelA pelE*) derivative of AC4150	He and Collmer 1990
CUCPB5030	*outD*::Tn*phoA* derivative of CUCPB5006	Bauer *et al.* 1994
CUCPB5045	*hrpN$_{Ech}$546*::Tn*5-gusA1* derivative of CUCPB5006	This work
CUCPB5046	*hrpN$_{Ech}$439*::Tn*5-gusA1* derivative of CUCPB5006	This work
CUCPB5063	*hrpN$_{Ech}$546*::Tn*5-gusA1* derivative of CUCPB5030	This work
CUCPB5049	*hrpN$_{Ech}$439*::Tn*5-gusA1* derivative of AC4150	This work
Erwinia amylovora		
Ea321	Wild type.	ATCC 49947
Ea321T5	*hrpN$_{Ea}$*::Tn*5*tac1 derivative of Ea321	Wei *et al.* 1992
Plasmids and phage		
pBluescript II SK(−)	Amp^r	Stratagene, La Jolla, CA
pCPP19	Cosmid vector, Sp^r/Sm^r	D. W. Bauer
pUC119	Amp^r plasmid vector	Vieira and Messing 1987
pSE280	Amp^r plasmid vector with superpolylinker downstream of tac promoter	Brosius 1989
pCPP2030	pCPP19 carrying *E. chrysanthemi* DNA hybridizing with *E. amylovora hrp* genes in pCPP1033	Bauer *et al.* 1994
pCPP1084	pBluescript M13+ carrying *hrpN$_{Ech}$* on 1.3-kb *Hin*dIII fragment	Wei *et al.* 1992
pCPP2157	pCPP19 carrying *E. chrysanthemi* DNA hybridizing with *E. amylovora hrpN*	This work
pCPP2142	8.3-kb *Sal*I subclone from pCPP2157 in pUC119	This work
pCPP2141	3.1-kb *Pst*I subclone from pCPP2157 in pBluescript II SK(−) *hrpN$_{Ech}$* in the orientation opposite that of the vector *lac* promoter	This work
pCPP2172	3.1-kb *Pst*I subclone from pCPP2157 in pBluescript II SK(−) *hrpN$_{Ech}$* in same orientation as vector *lac* promoter	This work
pCPP2174	1.0-kb *hrpN$^{*}_{Ech}$* polymerase chain reaction product cloned in *Nco*I-*Hin*dIII sites of pSE280	This work
λ::Tn*5-gusA1*	Tn*5* derivative for generating transcriptional fusions with *uidA* reporter; Kan^r, Tet^r	Sharma and Signer 1990

[a] Amp^r = ampicillin resistance; Kan^r = kanamycin resistance; Nal^r = nalidixic acid resistance; Sm^r = streptomycin resistance; Sp^r = spectinomycin resistance; Tet^r = tetracycline resistance.

the size of the macerated lesions that do develop. This pattern contrasts with that observed in mutations affecting Pel isozymes and the Out pathway. Virulence, as measured by maceration, is merely reduced by individual *pel* mutations, whereas it is abolished by *out* mutations. This is because multiple Pel isozymes (and possibly other enzymes) contribute quantitatively to virulence, but all of the Pel isozymes appear to be dependent on the Out pathway for secretion from the bacterial cell. The simplest interpretation of the observations with *E. chrysanthemi hrp* mutants is that $HrpN_{Ech}$ is the only protein traveling the Hrp pathway that has a detectable effect on the interaction of *E. chrysanthemi* EC16 with the plants tested.

The primacy of $HrpN_{Ech}$ in the *E. chrysanthemi* Hrp system is further supported by the observations that $hrpN_{Ech}$ mutants failed to elicit necrosis in any of the several plants tested and that all plants responding with apparent hypersensitivity to $HrpN^{+}_{Ech}$ strains also responded to isolated $HrpN_{Ech}$. Several of the plants sensitive to $HrpN_{Ech}$ are also susceptible to bacterial soft rots. This is particularly significant for African violet, whose interactions with *E. chrysanthemi* have been extensively studied (Barras *et al.* 1994). Thus, $HrpN_{Ech}$ elicits HR-like responses in plants that are susceptible to *E. chrysanthemi* infections under appropriate environmental conditions. The significance of this for the wide host range of the bacterium requires further investigation, and virulence tests with $hrpN_{Ech}$ mutants and additional susceptible plants are needed to determine the general importance of $HrpN_{Ech}$ and the Hrp system in *E. chrysanthemi.* For example, our present data do not address the possibility that other proteins secreted by the Hrp pathway, which are not elicitors of the HR in the plants we tested, may contribute to pathogenesis in hosts other than witloof chicory.

An important question is whether bacteria expressing heterologous harpins will be altered in pathogenicity. The *hrpN* genes of *E. chrysanthemi* and *E. amylovora* are particularly attractive for experiments addressing this because of the similarity of the harpins and the dissimilarity of the diseases produced by these bacteria. Unfortunately, attempts to restore the HR phenotype to *E. chrysanthemi* and *E. amylovora hrpN* mutants with heterologous $hrpN^{+}$ subclones failed. Since the *hrpN* genes in each subclone successfully complemented *hrpN* mutations in homologous bacteria and were expressed in heterologous bacteria, the problem is most likely the secretion of the harpins by heterologous Hrp systems. A similar problem has been encountered with heterologous secretion of Pel and cellulase via the Out pathway in *E. chrysanthemi* and *E. carotovora,* species that are more closely related to each other in this rather heterogeneous genus than *E. chrysanthemi* and *E. amylovora* are (He *et al.* 1991; Py *et al.* 1991).

In conclusion, two classes of proteins contribute to the pathogenicity of *E. chrysanthemi*—a single harpin and a battery of plant cell wall–degrading pectic enzymes. The observation that such a highly pectolytic organism also produces a harpin suggests the fundamental importance of harpins in the pathogenicity of gram-negative bacteria. The observation that an $hrpN_{Ech}$::Tn*5-gusA1* mutation reduced the ability of a fully pectolytic strain of *E. chrysanthemi* to initiate lesions in susceptible chicory leaves, but did not reduce the size of lesions that did devel p, suggests that $HrpN_{Ech}$ contributes specifically to an early stage of pathogenesis. An attractive possibility is that $HrpN_{Ech}$ releases nutrients to the apoplast for bacterial nutrition before the *pel* genes are fully expressed (Collmer and Bauer 1994). Patterns of *pel* and $hrpN_{Ech}$ expression *in planta* will likely yield further clues to the role of the *E. chrysanthemi* harpin in soft-rot pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

Bacterial stains and plasmids are listed in Table 3. *E. chrysanthemi* was routinely grown in King's medium B (King *et al.* 1954) at 30° C, *E. coli* in LM medium (Hanahan 1983) at 37° C, and *E. amylovora* in Luria-Bertani medium at 28–30° C. The following antibiotics were used in selective media in the amounts indicated (in µg/ml), except where noted: ampicillin (100), kanamycin (50), spectinomycin (50), and streptomycin (25).

General DNA manipulations.

Plasmid DNA manipulations, colony blotting, and Southern blot analyses were performed by standard techniques (Sambrook *et al.* 1989). Deletions for sequencing were constructed with the Erase-a-Base kit (Promega, Madison, WI). Double-stranded DNA sequencing templates were prepared with Qiagen Plasmid Mini Kits (Chatsworth, CA). Sequencing was performed with the Sequenase Version 2 kit (U.S. Biochemical, Cleveland, OH). The Tn*5-gusA1* insertion points were determined on an Automated DNA Sequencer (model 373A, Applied Biosystems, Foster City, CA) by the Cornell Biotechnology Center. DNA sequences were analyzed with the Genetics Computer Group Sequence Analysis Software Package (Devereaux *et al.* 1984). Comparison of $HrpN_{Ech}$ and $HrpN_{Ea}$ by the Gap program was done with a gap weight of 5.0 and a gap length weight of 0.3. Marker exchange mutagenesis was performed as previously described (Bauer *et al.* 1994). The oligonucleotide used to determine the location of Tn*5-gusA1* insertions in $hrpN_{Ech}$ was TGACCTGCAGCC-AAGCTTTCC. The oligonucleotide used as the first primer to amplify the $hrpN_{Ech}$ ORF and introduce an *Nco*I site at the 5′ end of the gene was AGTACCATGGTTATTACGATCAAA-GCGCAC; the one used as the second primer to introduce an *Xho*I site at the 3′ end of the gene was AGATCTCGAGGG-CGTTGGCCAGCTTACC. Primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Protein manipulations.

$HrpN_{Ech}$ was purified from *E. coli* DH5α(pCPP2172) cultures grown at 30° C to stationary phase in 50 ml of Terrific Broth (Sambrook *et al.* 1989) supplemented with ampicillin at a concentration of 200 µg/ml. Cells were lysed by lysozyme treatment and sonication as previously described (Sambrook *et al.* 1989). The lysate pellet was washed twice with 9 vol of lysis buffer containing 0.5% Triton X-100 and 10 mM EDTA, pH 8.0, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF); the lysate was reharvested each time by centrifugation at 12,000 × *g* for 15 min. The pellet was resuspended in 2.0 ml of lysis buffer containing 0.1 mM PMSF, dissolved by the addition of 2.5 ml of 8 M guanidine-HCl in lysis buffer, and then diluted with 5.0 ml of water. The protein solution was dialyzed in SpectraPor #1 dialysis tubing against 2.0 liters of 5 mM morpholinoethanesulfonic acid (MES), pH 6.5, containing 0.05 mM PMSF. The precipitate that formed dur-

ing dialysis and the solution were centrifuged for 15 min at 4,300 × g. The pellet was washed once with 10 ml of a solution containing 5 mM MES, pH 6.5, and 0.1 mM PMSF and then resuspended in 2.0 ml of the same buffer. Protein concentrations of homogeneous suspensions were determined following dissolution in the reagents of the dye-binding assay of Bradford (1976). Proteins in crude cell lysates or following purification were resolved by electrophoresis through an SDS 12% polyacrylamide gel and visualized by staining with Coomassie Brilliant Blue R. The N-terminal sequence of purified $HrpN_{Ech}$ was determined at the Cornell University Biotechnology Program Protein Analysis Facility.

Plant assays.

For HR assays, tobacco (*Nicotiana tabacum* L. cv. Xanthi), tomato (*Lycopersicon esculentum* Mill. cv. Sweet 199), pepper (*Capsicum annuum* L. cv. Sweet Hungarian), African violet (*Saintpaulia ionantha* H. Wendl. cv. Paris), petunia (*Petunia grandiflora* Juss. cv. Blue Frost), pelargonium (*Pelargonium hortorum* Bailey), winter squash (*Cucurbita maxima* Duchesne), and zinnia (*Zinnia elegans* Jacq.) plants were grown under greenhouse conditions or purchased at a local garden shop and then maintained in the laboratory at room temperature, with incident daylight supplemented with a 500-W halogen lamp. Witloof chicory (*Cichorium intybus* L.) was purchased as "Belgian endive" heads from a local supermarket. Bacterial inoculum was prepared and delivered as previously described (Bauer *et al.* 1994). Briefly, to assay soft-rot pathogenesis, 5 µl of inoculum was applied to a small wound in detached chicory leaves; to assay for HR elicitation, inoculum was infiltrated with a needle-less plastic syringe into leaves on plants.

ACKNOWLEDGMENTS

We thank Kent Loeffler for photography. This work was supported by NRI Competitive Grants Program/USDA grants 91-37303-6321 (AC), 94-37303-0734 (AC), and 91-37303-6430 (SVB).

LITERATURE CITED

Arlat, M., Van Gijsegem, F., Huet, J. C., Pernollet, J. C., and Boucher, C. A. 1994. PopA1, a protein which induces a hypersensitive-like response on specific *Petunia* genotypes, is secreted via the Hrp pathway of *Pseudomonas solanacearum*. EMBO J. 13:543-553.

Barras, F., Van Gijsegem, F., and Chatterjee, A. 1994. Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. Annu. Rev. Phytopathol. 32:201-234.

Bauer, D. W., Bogdanove, A. J., Beer, S. V., and Collmer, A. 1994. *Erwinia chrysanthemi hrp* genes and their involvement in soft rot pathogenesis and elicitation of the hypersensitive response. Mol. Plant-Microbe Interact. 7:573-581

Bonas, U. 1994. *hrp* genes of phytopathogenic bacteria. Pages 79-98 in: Current Topics in Microbiology and Immunology. Vol. 192. Bacterial Pathogenesis of Plants and Animals: Molecular and Cellular Mechanisms. J. L. Dangl, ed. Springer-Verlag, Berlin.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochem. 92:248-254.

Brosius, J. 1989. Superpolylinkers in cloning and expression vectors. DNA 8:759-777.

Burkholder, W. H., McFadden, L. A., and Dimock, A. W. 1953. A bacterial blight of chrysanthemums. Phytopathology 43:522-526.

Chatterjee, A. K., Thurn, K. K., and Feese, D. A. 1983. Tn*5* induced mutations in the enterobacterial phytopathogen *Erwinia chrysanthemi*. Appl. Environ. Microbiol. 45:644-650.

Collmer, A., and Bauer, D. W. 1994. *Erwinia chrysanthemi* and *Pseudomonas syringae*: Plant pathogens trafficking in virulence proteins. Pages 43-78 in: Current Topics in Microbiology and Immunology. Vol. 192. Bacterial Pathogenesis of Plants and Animals: Molecular and Cellular Mechanisms. J. L. Dangl, ed. Springer-Verlag, Berlin.

Conover, W. J. 1980. Practical Nonparametric Statistics. 2d ed. John Wiley and Sons, New York.

Devereaux, J., Haeberli, P., and Smithies, O. 1984. A comprehensive set of sequence analysis programs for the VAX. Gene 12:387-395.

Grant, S. G. N., Jessee, J., Bloom, F. R., and Hanahan, D. 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. USA 87: 4645-4649.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166:557-580.

He, S. Y., and Collmer, A. 1990. Molecular cloning, nucleotide sequence and marker-exchange mutagenesis of the exo-poly-α-D-galacturonosidase-encoding *pehX* gene of *Erwinia chrysanthemi* EC16. J. Bacteriol. 172:4988-4995.

He, S. Y., Huang, H.-C., and Collmer, A. 1993. *Pseudomonas syringae* pv. *syringae* $harpin_{Pss}$: A protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. Cell 73:1255-1266.

He, S. Y., Lindeberg, M., Chatterjee, A. K., and Collmer, A. 1991. Cloned *Erwinia chrysanthemi out* genes enable *Escherichia coli* to selectively secrete a diverse family of heterologous proteins to its milieu. Proc. Natl. Acad. Sci. USA 88:1079-1083.

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Med. 22:301-307.

Kiraly, Z. 1980. Defenses triggered by the invader: Hypersensitivity. Pages 201-224 in: Plant Disease: An Advanced Treatise. Vol. 5. J. G. Horsfall and E. B. Cowling, eds. Academic Press, New York.

Klement, Z. 1963. Rapid detection of pathogenicity of phytopathogenic pseudomonads. Nature 199:299-300.

Klement, Z. 1982. Hypersensitivity. Pages 149-177 in: Phytopathogenic Prokaryotes. Vol. 2. M. S. Mount and G. H. Lacy, eds. Academic Press, New York.

Klement, Z., Farkas, G. L., and Lovrekovich, L. 1964. Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathology 54:474-477.

Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. 1986. Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity of bean plants and hypersensitivity on nonhost plants. J. Bacteriol. 168: 512-522.

Michiels, T., Wattiau, P., Brasseur, R., Ruysschaert, J.-M., and Cornelis, G. 1990. Secretion of Yop proteins by Yersiniae. Infect. Immun. 58: 2840-2849.

Pearson, W. R., and Lipman, D. J. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA 85:2444-2448.

Py, B., Salmond, G. P. C., Chippaux, M., and Barras, F. 1991. Secretion of cellulases in *Erwinia chrysanthemi* and *E. carotovora* is species-specific. FEMS Microbiol. Lett. 79:315-322.

Salmond, G. P. C. 1994. Secretion of extracellular virulence factors by plant pathogenic bacteria. Annu. Rev. Phytopathol. 32:181-200.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2d ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sharma, S. B., and Signer, E. R. 1990. Temporal and spatial regulation of the symbiotic genes of *Rhizobium meliloti in planta* revealed by transposon Tn*5*-*gusA*. Genes Dev. 4:344-356.

Turner, J. G., and Novacky, A. 1974. The quantitative relation between plant and bacterial cells involved in the hypersensitive reaction. Phytopathology 64:885-890.

Van Gijsegem, F., Genin, S., and Boucher, C. 1993. Evolutionary conservation of pathogenicity determinants among plant and animal pathogenic bacteria. Trends Microbiol. 1:175-180.

Vieira, J., and Messing, J. 1987. Production of single-stranded plasmid DNA. Methods Enzymol. 153:3-11.

Wei, Z.-H., and Beer, S. V. 1993. HrpI of *Erwinia amylovora* functions in secretion of harpin and is a member of a new protein family. J. Bacteriol. 175:7958-7967.

Wei, Z.-M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A., and Beer, S. V. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science 257:85-88.

Willis, D. K., Rich, J. J., and Hrabak, E. M. 1991. *hrp* genes of phytopathogenic bacteria. Mol. Plant-Microbe Interact. 4:132-138.

RESULTS

RsmA$^-$ mutants f *E. carotovora* subsp. *carotovora* elicit responses in tobacco leaves that resemble the HR.

Previously (Chatterjee et al. 1995; Cui et al. 1995), we have described the isolation procedure as well as some of the characteristics of *E. carotovora* subsp. *carotovora* strain AC5070, the mini-Tn*5*-Km insertion RsmA$^-$ mutant (*rsm* = *r*egulator of *s*econdary *m*etabolites). Since AC5070 overproduces pectate lyases, polygalacturonases, protease, and cellulase, and is hypervirulent, it was of interest to examine the responses it could elicit in tobacco leaves, wherein wild-type *E. carotovora* subsp. *carotovora* does not cause tissue necrosis in 24 to 48 hr. As shown in Figure 1, cells of AC5070 infiltrated into tobacco leaves produced symptoms similar to those caused by *P. syringae* pv. *pisi*, known to elicit the HR. The lowest concentration of AC5070 that elicited an HR-like response was approximately 2×10^8 cells/ml. The visible symptoms, i.e., water soaking followed by tissue collapse, appeared within 24 h after the infiltration. By 24 h the inoculation sites developed necrosis, culminating in tissue desiccation. These responses, as in the typical HR, invariably remained confined to the area infiltrated with bacterial cells. Infiltration with cells of RsmA$^+$ *E. carotovora* subsp. *carotovora* grown in Luria-Bertani (LB) agar did not produce visible lesions; however, after 5 to 6 days the infiltrated sites became chlorotic.

By ethyl methane sulfonate (EMS) mutagenesis of *E. carotovora* subsp. *carotovora* strain AC5006, we isolated a mutant, AC5041, that, like AC5070, overproduces pectate lyases, polygalacturonases, protease, and cellulase (Fig. 2). In addition, the mutant is hypervirulent in that it caused more severe maceration in celery petioles than the parent RsmA$^+$ strain (Fig. 3). The derepressed mutant, AC5041, but not its parent strain, induced the HR-like response in tobacco leaves (data not shown).

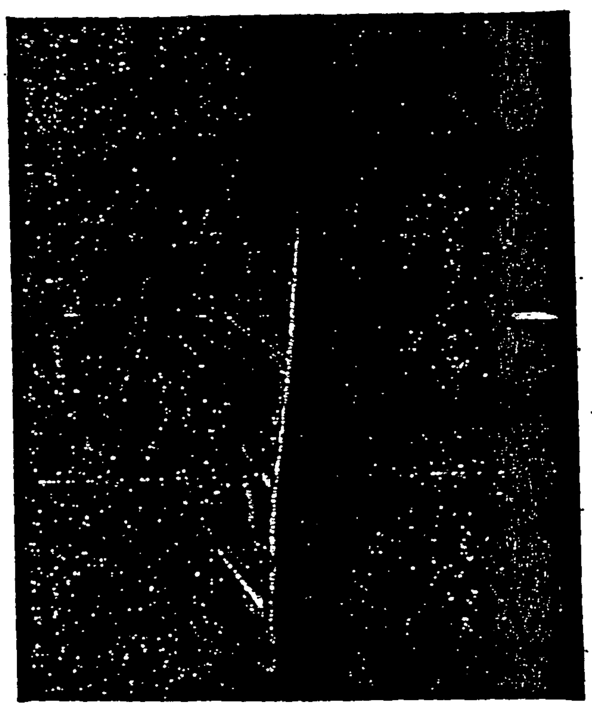


Fig. 1. Symptoms produced in tobacco leaves by *Erwinia carotovora* subsp. *carotovora* AC5047 and its RsmA$^-$ mutant, AC5070. Cell suspensions containing about 2×10^8 CFU/ml were infiltrated into each leaf segment. A, AC5047; B, AC5070; C, *Pseudomonas syringae* pv. *pisi* Psp1; and D, water. Picture was taken 24 h after infiltration.

Prevention of the HR-like response.

It has been reported that *P. syringae* pv. *pisi* prevents the HR when it is preinoculated in tobacco leaves at a lower concentration (5×10^5) and later challenged with an HR-inducing concentration (5×10^6) at the same site (Novacky et al. 1973). Similarly, we have noticed that preinfiltration of tobacco leaves with AC5070 (10^5 CFU/ml) prevented the appearance of water soaking and necrosis upon reinoculation at the same

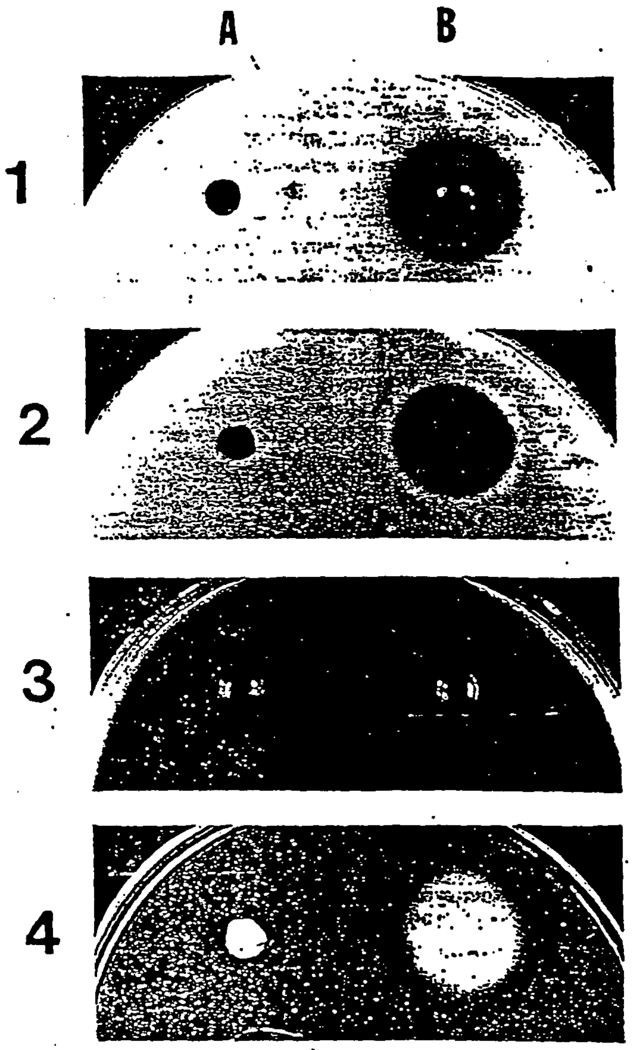


Fig. 2. Agarose plate assays for 1, pectate lyase (Pel); 2, polygalacturonase (Peh); 3, protease (Prt); and 4, cellulase (Cel) activities of *Erwinia carotovora* subsp. *carotovora* AC5006 (A) and its RsmA$^-$ mutant AC5041 (B). Bacteria were grown in salts–yeast extract–glycerol medium to saturation. Culture supernatants were diluted twofold in 10 mM Tris-HCl (pH 7.0) buffer and 5 µl of the diluted samples were used for the Pel, Peh, and Cel assays. Thirty microliters of undiluted samples were used for the Prt assay.

site with AC5070 or *P. syringae* pv. *pisi* (Fig. 4). After the preinoculation, about 2 × 10[8] cells of AC5070 were introduced at different intervals. The ability f preinoculated cells to inhibit the HR-like response was apparent by 12 h after inoculation (data not shown), and by 24 h production of the response was completely suppressed.

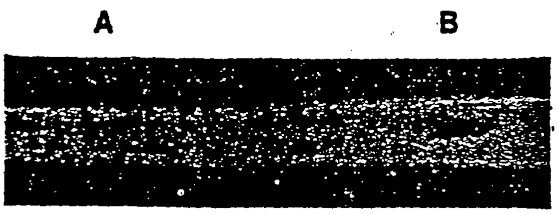


Fig. 3. Maceration of celery petioles induced by *Erwinia carotovora* subsp. *carotovora* AC5006 (A) and its RsmA⁻ mutant AC5041 (B). About 2 × 10[8] bacterial cells suspended in water were injected into each inoculation site. Inoculated petioles were covered with petroleum jelly and incubated in a moist chamber at 25°C for 24 h.

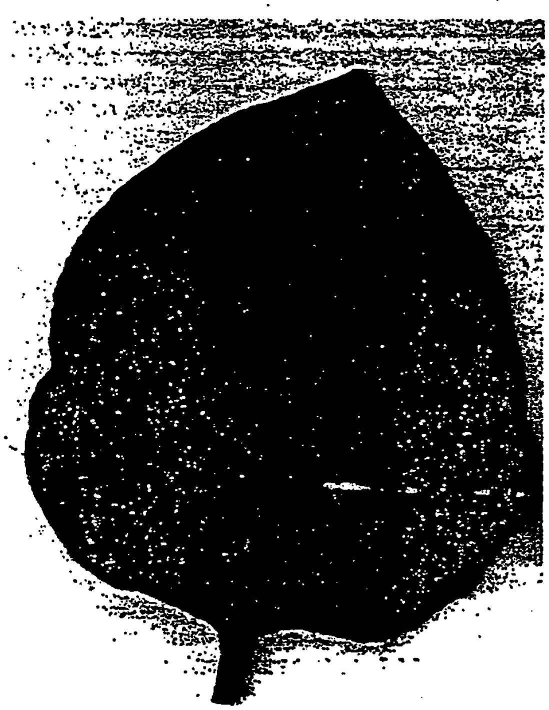


Fig. 4. Prevention of the hypersensitive response symptoms in tobacco leaf by the RsmA⁻ mutant of *Erwinia carotovora* subsp. *carotovora*, AC5070. Leaf segments were infiltrated with A, water at 0 h; B, *Pseudomonas syringae* pv. *pisi* Psp1 (5 × 10[6] CFU/ml) at 24 h; C, AC5070 (2 × 10[8] CFU/ml) at 24 h; D, AC5070 (2 × 10[8] CFU/ml) at 0 h; E, AC5070 (10[5] CFU/ml) at 0 h; F, AC5070 (10[5] CFU/ml) at 0 h and challenged with Psp1 (5 × 10[6] CFU/ml) after 24 h; G, AC5070 (10[5] CFU/ml) at 0 h and challenged with AC5070 (2 × 10[8] CFU/ml) after 24 h; and H, Psp1 (5 × 10[6] CFU/ml) at 0 hour. Leaf was photographed 48 h after infiltration.

RsmA⁻ mutants of *E. carotovora* subsp. *carotovora* elicit the HR-like response in the absence of the cell density sensing signal, OHL.

OHL and its structural analogs are required for the expression of many phenotypes in different bacteria (Fuqua et al. 1994; Salmond et al. 1995; Swift et al. 1994). In *E. carotovora* subsp. *carotovora*, OHL controls extracellular enzyme production, pathogenicity, and production of the antibacterial antibiotic, carbapenem (Bainton et al., 1992; Chatterjee et al. 1995; Jones et al. 1993; Pirhonen et al. 1993). We had previously demonstrated that exoenzyme overproduction and pathogenicity occurred in the absence of OHL in the RsmA⁻ mutant, AC5070 (Chatterjee et al. 1995). To find out if the mutants could elicit the HR-like response in the absence of this cell density sensing signal, we examined the responses induced by OHL-deficient derivatives of the RsmA⁻ strains. We made the EMS-induced RsmA⁻ mutant OHL deficient by replacing *ohlI*⁺ (previously designated as *hslI*⁺) allele required for OHL biosynthesis, with *ohlI*-MudI by marker exchange, as we had done with AC5070 (Chatterjee et al. 1995). AC5090 and AC5093, the derivatives of AC5070 and AC5041, respectively, do not produce OHL, as indicated by the Lux bioassay (Chatterjee et al. 1995; data not shown). Figure 5 shows that AC5090 and AC5093 elicited reactions in tobacco leaves that were very similar to those produced by the parent strains as well as by *P. syringae* pv. *pisi*.

The RsmA⁻ mutants overexpress $hrpN_{Ecc}$, a locus presumed to specify an elicitor of the HR.

Recent studies by S. V. Beer, A. Collmer, and their associates demonstrated that *hrpN* genes of *E. amylovora* and *E. chrysanthemi* encode elicitors of the HR and raised the possi-

Fig. 5. Elicitation of the hypersensitive-like response in tobacco leaves by RsmA⁻ mutants of *Erwinia carotovora* subsp. *carotovora* and their OhlI⁻ derivatives. Leaf segments were infiltrated with 2 × 10[8] CFU/ml of bacterial cells. A, water; B, AC5093 (RsmA⁻, Ohl⁻); C, AC5090 (RsmA⁻, Ohl⁻); D, *Pseudomonas syringae* pv. *pisi* Psp1; E, AC5041 (RsmA⁻, Ohl⁺); and F, AC5070 (RsmA⁻, Ohl⁺). Picture was taken 24 h after infiltration.

```
HrpNEcc   MLNSLGG--GASLQITIKA-GGNGGLFPSQSSQNG--GSPSQSAFGGQRS   45
HrpNEch   MQITIKA--HIGGDLGVSG-LGLGA--QGLKGLNS--AASSLGSSVDKLS   43
HrpNEa    MSLNTSGLGASTMQISIGGAGGNNGLLGTSRQNAGLGGNSALGLGGGNQN   50
          *  .  .    . .... .   * ..   .  .  ..  .... .   .. .

HrpNEcc   NIAEQLSDIMTTMMFM------GSMMGGGMSGGLGGLGSSLGGLGGGL--   87
HrpNEch   STIDKLTSALTSMMF-----------GGALAQGLGASSKGLG--------   74
HrpNEa    DTVNQLAGLLTGMMMMMSMMGGGGLMGGGLGGGLGNGLGGSGGLGEGLSN  100
          .. ..*.. .*.**.                **... ***.   . *

HrpNEcc   -LGGGLGGGLGSSLGSGLGSALGGGLGGALGAGM----------------  120
HrpNEch   -MSNQLGQSFGNG-AQGASNLLSVPKSG--GDAL----------------  104
HrpNEa    ALNDMLGGSL-NTLGSKGGNNTTSTTNSPLDQALGINSTSQNDDSTSGTD  149
           ... ** .. .. .   ..   .   ..  ....

HrpNEcc   NAMNPSAMMGSLL---FSALEDLLGGGMSQQQGGLFGNKQPSSPEISAYT  167
HrpNEch   SKMFDKAL-DDLL---GHDTVTKLTNQSNQLANSMLNASQMTQGNMNAFG  150
HrpNEa    STSDSSDPMQQLLKMFSEIMQSLFGDGQDGTQGSSSGGKQPTEGEQNAYK  199
          .   ..    **          . ...  .  ...   ...*  .   .*.

HrpNEcc   QGVNDNLSAILGNGLSQTKG--------------QTSPLQLGNNGLQGLS  203
HrpNEch   SGVNNALSSILGNGLGQSMS--------------GFSQPSLGAGGLQGLS  186
HrpNEa    KGVTDALSGLMGNGLSQLLGNGGLGGGQGGNAGTGLDGSSLGGKGLQNLS  249
           **...**...****.*  .                .   **. ***.**

HrpNEcc   GAGAFNQLGSTLGMSVGQKAGLQELNNISTHNDSPTRYFVDKEDRGMAKE  253
HrpNEch   GAGAFNQLGNAIGMGVGQNAALSALSNVSTHVDGNNRHFVDKEDRGMAKE  236
HrpNEa    GPVDYQQLGNAVGTGIGMKAGIQALNDIGTHRHSSTRSFVNKGDRAMAKE  299
          *. ...***...* ..* .*.. .*....** .. .* **.*.**.****

HrpNEcc   IGQFMDQYPEVFGKAEYQKDNWQTAKQEDKSWAKALSKPDDDGMTKGSMD  303
HrpNEch   IGQFMDQYPEIFGKPEYQKDGWSSPKTDDKSWAKALSKPDDDGMTGASMD  286
HrpNEa    IGQFMDQYPEVFGKPQYQKGPGQEVKTDDKSWAKALSKPDDDGMTPASME  349
          **********.***..***.  . .  * .****************  .**.

HrpNEcc   KFMKAVGMIKSAIRGDTGNTNLSARGNGGASLGIDAAMIGDRIVNMGLKK  353
HrpNEch   KFRQAMGMIKSAVAGDTGNTNLNLRGAGGASLGIDAAVVGDKIANMSLGK  336
HrpNEa    QFNKAKGMIKRPMAGDTGNGNL------------------QHAVPVVLRW  381
          .* .* ****...  *****.**                  . . .  *

HrpNEcc   LSS-   356
HrpNEch   LANA   340
HrpNEa    VLMP   385
```

Fig. 6. Alignment of deduced amino acid sequence of *$hrpN_{Ecc}$* of *Erwinia carotovora* subsp. *carotovora* strain Ecc71 ($HrpN_{Ecc}$) with those of *E. chrysanthemi* EC16 ($HrpN_{Ech}$) and *E. amylovora* Ea321 ($HrpN_{Ea}$). Asterisks indicate identical amino acids; single dots indicate conservative substitutions. Numbers at R right indicate amino acid positions in each protein.

bility that *hrp* genes including *hrpN* may also occur in other *Erwinia* species (Bauer et al. 1994; Bauer et al. 1995; Laby and Beer 1992; Wei et al. 1992). Indeed, Southern blot hybridization under moderate stringency conditions with *hrpN* DNA of *E. chrysanthemi* (EC16) (Bauer et al. 1995) as the probe disclosed the presence of *hrpN* sequences in *E. carotovora* subsp. *carotovora* strain Ecc71 (data not shown). Subsequently, by screening a library of Ecc71 with the *hrpN* DNA of *E. chrysanthemi*, several clones possessing homologous DNA were identified; the corresponding Ecc71 sequences are tentatively designated as $hrpN_{Ecc}$. Sequence analysis of the DNA segment that specifically hybridized with the *hrpN* DNA of *E. chrysanthemi* revealed an 1,068-bp open reading frame whose predicted product has 72.1% similarity and 53.4% identity with the deduced product of *hrpN* of *E. chrysanthemi*, and 66.6% similarity and 50.8% identity with the predicted product of *hrpN* of *E. amylovora* (Fig. 6).

Northern (RNA) blot analysis was performed with total RNA preparations from the wild-type strain Ecc71, the RsmA⁻ mutants, AC5041 and AC5070, and their RsmA⁺ parents to ascertain if $hrpN_{Ecc}$ expression is derepressed in the RsmA⁻ strains. Bacteria were grown in SYG medium at 28°C to a Klett value of approximately 200 and used for total RNA isolation. A 700-bp *AccI-SmaI* internal fragment of the $hrpN_{Ecc}$ was used as the probe. The data (Fig. 7) revealed the presence of 1100-base transcripts in AC5070 and AC5041. By contrast, these transcripts were not detected with RsmA⁺ strains 71, AC5006 and AC5047. We should note that somewhat higher levels of $hrpN_{Ecc}$ transcripts were present in the mini-Tn*5*-Km insertion mutant (AC5070) than in the EMS-induced mutant (AC5041). We do not yet know the reason for this difference. It is possible that AC5041 produces a defective RsmA with a leaky activity, whereas the mini-Tn*5*-Km insertion mutant does not produce a functional RsmA. It is, however, clear that $hrpN_{Ecc}$ transcripts are substantially higher in AC5041 than in its RsmA⁺ parent, AC5006.

The *rsmA*⁺ allele suppresses elicitation of the HR-like response and expression of $hrpN_{Ecc}$.

We have previously described the cloning and characterization of the *rsmA* gene of *E. carotovora* subsp. *carotovora* strain Ecc71 (Chatterjee et al. 1995; Cui et al. 1995). A low-copy plasmid carrying this gene causes a severe attenuation of pathogenicity and suppresses extracellular enzyme production in *E. carotovora* subsp. *carotovora* and *E. c.* subsp. *atroseptica*; represses pathogenicity, exopolysaccharide production, flagellum production and motility, protease production, and elicitation of the HR by *E. amylovora*; and suppresses extracellular enzyme and antibiotic production by *E. carotovora* subsp. *betavasculorum* (Chatterjee et al. 1995; Cui et al. 1995; Mukherjee et al. 1996a, 1996b). In light of the large array of effects on phenotypes by *rsmA*, including induction of the HR by *E. amylovora*, it was deemed worthwhile to examine the effects of the *rsmA*⁺ DNA on elicitation of the HR-like response by the mutants. The plasmids pCL1920 and pAKC880 were transformed into AC5041 and AC5070 and the constructs were tested for induction of the HR-like response. Figure 8 shows that AC5041 and AC5070 carrying the cloning vector, pCL1920, elicited reactions in tobacco leaves similar to those caused by *P. syringae* pv. *pisi*. By contrast, there was no visible reaction in the leaf segment infiltrated with AC5041 or AC5070 carrying the RsmA⁺ plasmid, pAKC880. These results indicate that multiple copies of *rsmA* suppress elicitation of the HR-like response in tobacco leaves by AC5041 and AC5070.

Northern analysis was conducted to determine the effect of RsmA plasmid on $hrpN_{Ecc}$ transcription. The data (Fig. 9) show that high levels of $hrpN_{Ecc}$ transcripts were present in cells of AC5041 and AC5070 containing the cloning vector, pCL1920, but the transcripts were not detected in cells carrying the *rsmA* plasmid, pAKC880.

DISCUSSION

We previously reported that extracellular enzyme production as well as virulence are negatively regulated by *rsmA* in *E. carotovora* subsp. *carotovora* (Chatterjee et al. 1995; Cui et al. 1995; Mukherjee et al. 1996a, 1996b). For example, the inactivation of *rsmA* by a transposon resulted in overproduction of extracellular enzymes and hypervirulence. Moreover, unlike its parent, the RsmA⁻ mutant did not require the cell density sensing signal, OHL, for pathogenesis or extracellular enzyme production. In this report, we have shown that this RsmA⁻ mutant and an EMS-induced mutant of a similar phenotype elicited the HR-like response in tobacco leaves, and that the elicitation of this reaction was also not dependent upon OHL. Although we do not yet have direct evidence that the mutations in AC5041 and AC5070 are in the same gene, these strains possess similar phenotypes; e.g., they overproduce extracellular enzymes, they are hypervirulent, and OHL deficiency does not affect the expression of these traits. Moreover, the plasmid carrying *rsmA*⁺ DNA suppresses extracellular enzyme production, pathogenicity, and the elicitation

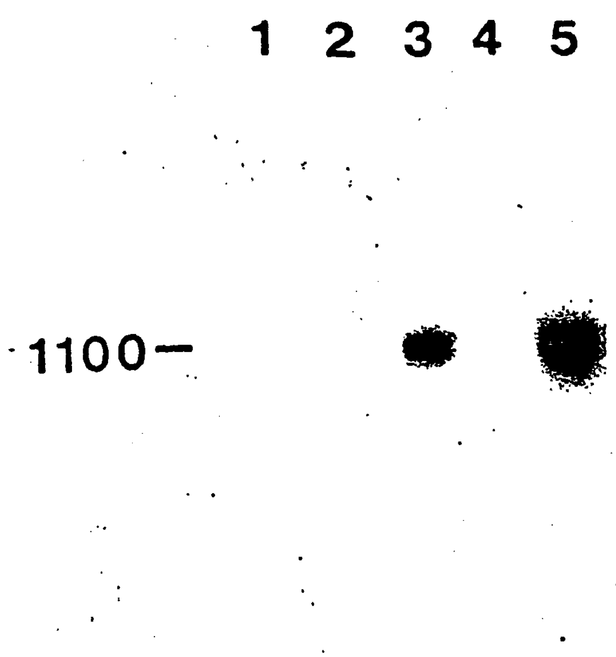


Fig. 7. Northern (RNA) blot analysis of $hrpN_{Ecc}$ mRNA of *Erwinia carotovora* subsp. *carotovora* strains. Each lane contained 20 µg of total RNA. Position of 1100-base transcript is indicated. Lane 1, Ecc71 (wild-type parent, RsmA⁺); lane 2, AC5006 (RsmA⁺); lane 3, AC5041 (RsmA⁻); lane 4, AC5047 (RsmA⁺); lane 5, AC5070 (RsmA⁻).

of the HR-like response by the mutants. Also, both the mutants express $hrpN_{Ecc}$ constitutively, although the transcript level is somewhat higher in AC5070 than in AC5041. As these mutants have similar phenotypes, we tentatively classified them as RsmA⁻.

The following lines of evidence strongly suggest that the mutants elicited a typical HR (Goodman and Novacky 1994): (i) the reaction was characterized by a rapid physiological activity (i.e., water movement or water soaking), tissue collapse followed by cell death (necrosis); (ii) the affected areas were limited to the region infiltrated with bacterial cells; (iii) these symptoms were indistinguishable from the symptoms developed by *P. syringae* pv. *pisi*, a bacterium known to elicit the typical HR in tobacco leaves; (iv) the response elicited by AC5070 was preventable upon previous infiltration of a low concentration of AC5070 cells and, similarly, prior inoculations with AC5070 cells prevented elicitation of the HR by *P. syringae* pv. *pisi*; and (v) while AC5070 and AC5041, their parent strains, and the wild-type strain possess $hrpN_{Ecc}$ sequences (data not shown), the expression of $hrpN_{Ecc}$ is derepressed only in the mutants, presumably leading to the production of high levels of a putative elicitor of the HR (see below).

Our observations support the idea that AC5070 and AC5041 produce an elicitor that triggers the HR-like response in tobacco leaves. We attribute the manifestation of this response with the mutants, but not with the parents, to the ability of the former to produce high constitutive levels of $HrpN_{Ecc}$, an exoenzyme, or both. With regard to the possible role of exoenzymes, it is perhaps significant that pectinases are known to generate elicitors of plant defense responses (Davis et al. 1984; Davis and Ausubel 1989; Keen 1992). Furthermore, Palva et al. (1993) have documented the activation of chitinases and glucanases in tobacco by exoenzyme-producing strains of *E. carotovora* subsp. *carotovora* but not by mutants deficient in exoenzyme production. Therefore, one could argue that pectinase overproduction by the RsmA⁻ mutants may induce defense reactions that could culminate in an HR-like response. The inability of the wild-type RsmA⁺ *E. carotovora* subsp. *carotovora* strain Ecc71 to elicit this response could be attributed to the lack of extracellular enzyme production in a nonhost tissue, i.e., in a tobacco leaf. However, the hypothesis implicating pectolytic enzymes as elicitors of the HR is difficult to reconcile with the finding of Bauer et al. (1994) that only those mutants of *E. chrysanthemi* that are deficient in major pectate lyases can elicit the HR.

In light of that finding and for the following reasons, we favor the hypothesis that induction of the HR-like response by the mutants may be due to the derepression of a gene encoding an elicitor, such as $HrpN_{Ech}$ or $HrpN_{Ea}$. Collmer and asso-

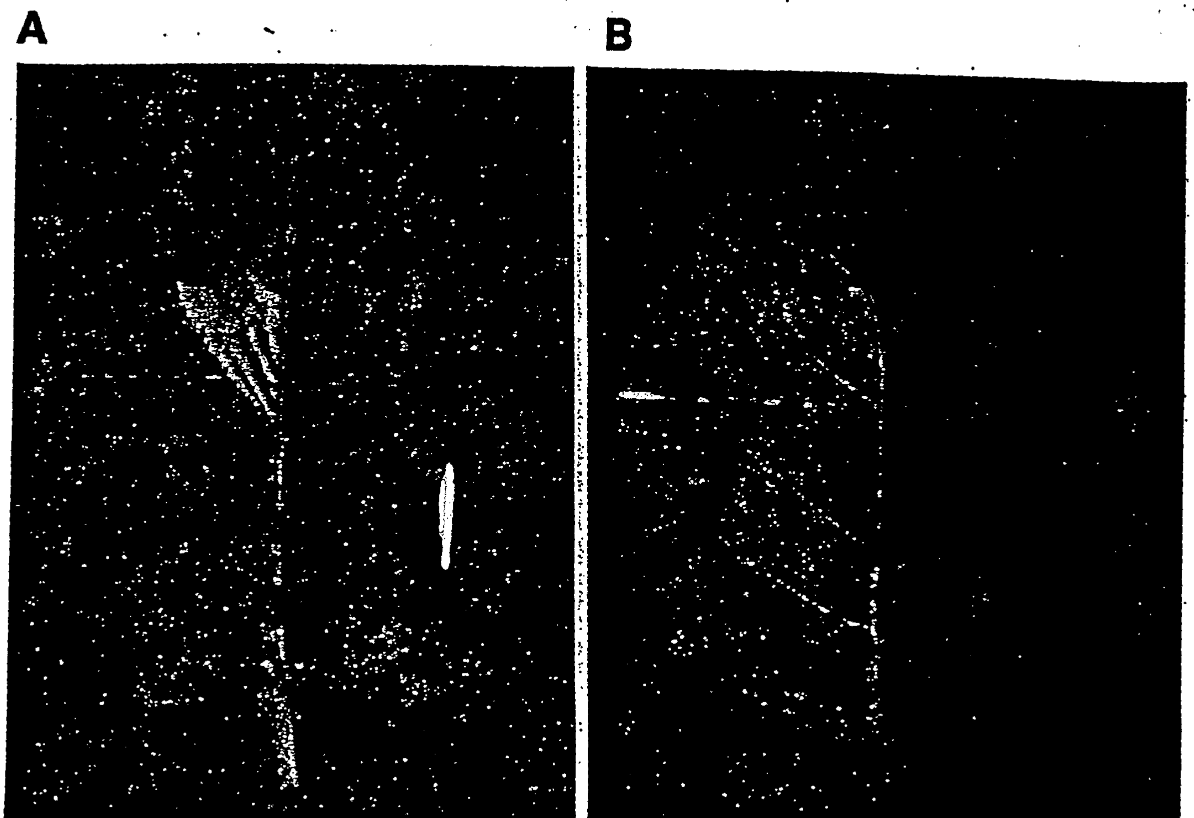


Fig. 8. Elicitation of an hypersensitive-like response in tobacco leaves by the RsmA⁻ mutants of *Erwinia carotovora* subsp. *carotovora* AC5041 (panel A) and AC5070 (panel B) carrying the *rsmA*⁺ plasmid, pAKC880, or the cloning vector, pCL1920. Bacterial suspensions containing about 2 × 10⁸ CFU/ml were infiltrated into each leaf segment. Panel A: A, *Pseudomonas syringae* pv. *pisi* Psp1; B, AC5041 carrying pAKC880; C, water; D, AC5041 carrying pCL1920. panel B: A, Psp1; B, AC5070 carrying pAKC880; C, water; D, AC5070 carrying pCL1920. Picture was taken 24 h after infiltration.

ciates (Bauer et al. 1994; Bauer et al. 1995) have discovered a gene specifying an elicitor of the HR in the soft-rotting bacterium *E. chrysanthemi*. The deduced sequence of HrpN$_{Ecc}$ presented here document the occurrence of a homolog of *E. chrysanthemi hrpN* in *E. carotovora* subsp. *carotovora* strain Ecc71. We have found that the mini-Tn*5*-Km induced RsmA$^-$ mutant as well as the EMS-induced derepressed mutant possess a substantial level of an approximately 1100-base transcript that specifically hybridizes with the *hrpN*$_{Ecc}$ DNA. By contrast, this transcript is barely detectable in the RsmA$^+$ strains. Moreover, the introduction of the *rsmA*$^+$ allele into the mutants severely reduces the levels of this transcript and concomitantly abolishes the ability to elicit the HR-like response. These observations indicate that transcription of *hrpN*$_{Ecc}$ is derepressed in the mutants, and that this derepression is due to the inactivation of *rsmA*. At the moment, since the genes for pectolytic enzymes and *hrpN*$_{Ecc}$ are both derepressed in the RsmA$^-$ mutants, we have to entertain the possibility that the pectolytic enzymes could also contribute to the hypersensitive necrosis of tobacco leaf tissue. Genetic and biochemical studies have been initiated to determine if *hrpN*$_{Ecc}$ and its putative product are solely responsible for the elicitation of the HR and to clarify the ramifications of *hrpN*$_{Ecc}$ regulation in compatible and incompatible interactions of *E. carotovora* subsp. *carotovora*.

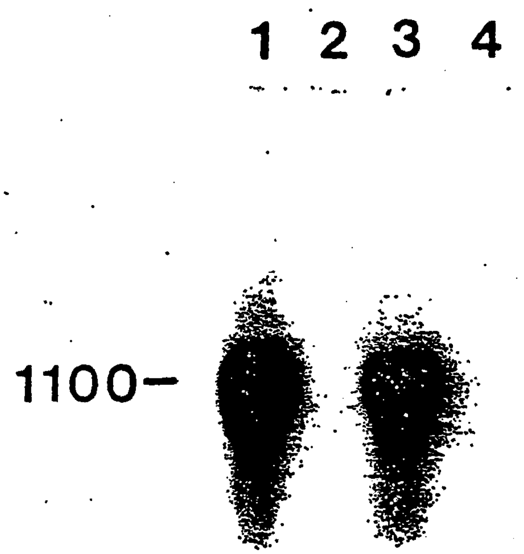


Fig. 9. Northern (RNA) blot analysis of *hrpN*$_{Ecc}$ mRNA of *Erwinia carotovora* subsp. *carotovora* RsmA$^-$ mutants AC5041 and AC5070 carrying the *rsmA*$^+$ plasmid, pAKC880, or the cloning vector, pCL1920. Each lane contained 20 µg of total RNA. The position of 1100-base transcript is indicated. Lane 1, AC5070 carrying pCL1920; lane 2, AC5070 carrying pAKC880; lane 3, AC5041 carrying pCL1920; lane 4, AC5041 carrying pAKC880.

MATERIALS AND METHODS

Bacterial strains and media.

Bacterial strains and plasmids are described in Table 1. *E. carotovora* subsp. *carotovora* strains were routinely grown in LB and *P. syringae* pv. *pisi* on King's B (King et al. 1954) agar media at 28°C. Minimal salts plus sucrose (0.2%) agar, nutrient gelatin (NG) agar, polygalacturonate-yeast extract agar (PYA) and salts-yeast extract-glycerol (SYG) media have been described previously (Barras et al. 1987; Chatterjee 1980; Murata et al. 1991). When required, antibiotics were added at the indicated concentrations in micrograms per milliliter: spectinomycin (Spc), 50; tetracycline (Tc), 10; Ampicillin (Ap), 50 and Kanamycin (Km), 50. The composition of agarose media for semiquantitative assays of enzymatic activities has been described in Chatterjee et al. (1995).

Enzyme assays.

The preparation of enzyme samples for assays as well as the assay procedures were described previously (Murata et al. 1991; Chatterjee et al. 1995). The volumes of enzyme samples used in the assays are indicated in the figure legends.

Bioluminescence assay for OHL.

The procedure described by Chatterjee et al. (1995) was followed.

Recombinant DNA techniques.

Standard procedures were followed in DNA isolation, transformation and electroporation of bacteria, restriction digests, gel electrophoresis, DNA ligation, and Southern blot analysis (Sambrook et al. 1989). Restriction and modifying enzymes were obtained from Promega Biotech (Madison, WI).

Isolation of RsmA$^-$ mutants.

The procedure used for the isolation of AC5070 by mini-Tn*5*-Km has been described (Chatterjee et al. 1995). AC5041 was isolated by EMS mutagenesis of AC5006. Mutagenesis was carried out according to the protocol of Miller (1972). The bacterial cells were incubated with EMS for a period that yielded less than 5% survival. The putative RsmA$^-$ mutants were identified by their ability to overproduce protease, cellulase, and pectolytic enzymes in agar plate assays (Chatterjee et al. 1995).

Inactivation of the *ohl* locus by MudI mutagenesis.

The plasmid, pAKC852, carrying the 9.7-kb *ohl*$^+$ DNA of *E. carotovora* subsp. *carotovora* strain Ecc71 was mutagenized with MudI1734 following the procedure of Castilho et al. (1984). Briefly, pAKC852 was transformed into the lysogenic *Escherichia coli* strain POI1734. The strain carrying the Ohl$^+$ plasmid was heat-induced to lyse. The lysate was used to transduce *E. coli* M8820, and the Tc^{r}Km^r transductants were screened for OHL production by means of the plate assay procedure described in Chatterjee et al. (1995). Plasmids were isolated from M8820 colonies that could no longer activate the *lux* operons in pHV200I.

Construction of bacterial strains by marker exchange.

The construction of AC5090, the Ohl$^-$ derivative of AC5070, has been described (Chatterjee et al. 1995). To isolate AC5093, the Ohl$^-$ mutant f AC5041, the plasmid (pAKC863) carrying inactivated *ohlI*-MudI was transferred into AC5041 by means of the h lper plasmid, pRK2013.

Transconjugants were selected on minimal salts plus sucrose agar supplemented with Km. Colonies that were Km^{r}Tc^s were tested for the Ohl phenotype. AC5093 was selected for further studies.

Plant tissue maceration.

The celery petiole assay was previously described (Murata et al. 1991). The extent of tissue maceration was estimated visually.

Infiltration of tobacco leaves.

Erwinia species were grown on LB agar and *P. syringae* pv. *pisi* was grown on King's B agar overnight at 28°C and cells were resuspended in water. Strains carrying plasmids were grown on LB agar containing spectinomycin and cells suspended in a 50 µg/ml spectinomycin solution in water. Young, fully expanded third and fourth leaves of about 8-week-old *Nicotiana tabacum* L. cv. Samsun were infiltrated with bacterial suspensions. Inoculated plants were incubated in a growth chamber at 27°C with a 14/10 h daylight regime and visually m nitored for reactions. For testing the prevention of the HR-like response, cells of AC5070 (10^5 CFU/ml) were infiltrated into tobacco leaves. The preinoculated areas were reinoculated with 2×10^8 CFU of AC5070 per ml or 5×10^6 CFU of *P. syringae* pv. *pisi* Psp1 per ml at desired intervals.

Cloning of *hrpN$_{Ecc}$* DNA and nucleotide sequence analysis.

The genomic library of *E. carotovora* subsp. *carotovora* strain Ecc71 in pLARF5 was screened by in situ colony hybridization with a 0.75-kb internal *Cla*I fragment of *hrpN* of *E. chrysanthemi* (Bauer et al. 1995). Two cosmids, pAKC921 and pAKC922, that hybridized with the probe were isolated. The subclones (pAKC923 and pAKC924, Table 1) carrying *hrpN* DNA were used for sequence analysis.

Unidirectional 5′ to 3′ deletions of pAKC924 were made and the overlapping deletions differing in size by approximately 200 bp were used for sequence analysis with the Sequenase System II (U.S. Biochemicals, Cleveland, OH). In addition, we used oligonucleotide primers to verify and complete the sequence of *hrpN$_{Ecc}$* with pAKC923 and pAKC924 DNAs as templates. Alignment of protein sequences was performed using the Genetics Computer Group, Inc. (Madison, WI) software program at the DNA Core facility on the University of Missouri-Columbia campus and the PC/GENE program (IntelliGenetics, Inc., Mountain View, CA). The sequence of *hrpN$_{Ecc}$* has been deposited at GenBank and has been assigned accession number L78834.

Northern blot analysis.

Bacterial cultures were grown to a value of approximately 200 Klett units at 28°C in SYG medium with or without

Table 1. Bacterial strains and plasmids

Bacteria	Relevant characteristics[a]	Reference or source
Erwinia carotovora subsp. *carotovora*		
71	Wild type	Zink et al. 1984
AC5006	Lac$^-$ mutant of 71	Murata et al. 1991
AC5041	RsmA$^-$, EMS mutant of AC5006	This study
AC5047	Nal^r derivative of AC5006	Chatterjee et al. 1995
AC5070	RsmA$^-$, mini-Tn5-Km mutant of AC5047, Km^r, Nal^r	Chatterjee et al. 1995
AC5090	Ohl$^-$ derivative of AC5070, RsmA$^-$, Km^r, Spc^r	Chatterjee et al. 1995
AC5093	Ohl$^-$ derivative of AC5041, RsmA$^-$, Km^r	This study
Pseudomonas syringae pv. *pisi*		
Psp1	Wild type	A. J. Novacky
Escherichia coli		
DH5α	*φ80lacZ ΔM15, Δ(lacZYA-argF), U169 hsdR17 recA1 endA1 thi-1*	BRL, Frederick, MD
HB101	*proA1 lacY hsdS20(rB$^-$ mB$^-$), recA56 rpsL20*	Zink et al. 1984
M8820	*Δ(proAB-argF-lacPOZYA)recA$^+$*	Castilho et al. 1984
POI1734	MudI1734::ara(Mu cts), *Δ(proAB-argF-lacIPOZYA)*	Castilho et al. 1984
VJS533	*araΔ(lac-proAB) rpsL φ80lacZ, ΔM15 recA56*	Gray and Greenberg 1992
Plasmids		
pAKC852	OhlI$^+$, Tc^r	Chatterjee et al. 1995
pAKC863	Derived from pAKC852, *ohlI*::MudI, Km^r, Tc^r	This study
pAKC880	RsmA$^+$, Spc^r	Cui et al. 1995
pAKC921	pLARF5 containing *hrpN$_{Ecc}$* from genomic library of Ecc71, Tc^r	This study
pAKC922	pLARF5 containing *hrpN$_{Ecc}$* from genomic library of Ecc71, Tc^r	This study
pAKC923	4.0-kb *Eco*RI fragment of pAKC921 containing *hrpN$_{Ecc}$* cloned into pSK$^+$, Ap^r	This study
pAKC924	1.4-kb *Eco*RI fragment of pAKC922 containing *hrpN$_{Ecc}$* cloned into pSK$^+$, Ap^r	This study
pCL1920	Spc^r	Lerner and Inouye 1990
pCPP2172	*hrpN$_{Ech}$*, Ap^r	Bauer et al. 1995
pLARF5	Tc^r	Keen et al. 1988
pRK415	Tc^r	Keen et al. 1988
pRK2013	Mob$^+$, Tra$^+$, Km^r	Figurski and Helinski 1979
pBluescript SK+	Ap^r	Stratagene, La Jolla, CA
pHV200	8.8-kb *lux* DNA in pBR322, Ap^r	Gray and Greenberg 1992
pHV200I	Frameshift mutation of luxI in pHV200, Ap^r	Pearson et al. 1994

[a] Uncommon abbreviations: EMS = ethyl methane sulfonate; Ohl = *N*-(3-oxohexanoyl)-L-homoserine lactone, designated as Hsl in our previous publications; *rsmA* = regulator of secondary metabolites; *hrpN$_{Ecc}$* = *E. carotovora* subsp. *carotovora* DNA fragment carrying a *hrpN$_{Ech}$* homolog (Bauer et al. 1995).

spectinomycin. The procedures for RNA isolation and Northern blot analysis described in Chatterjee et al. (1991) and Liu et al. (1993) were followed. A 0.7-kb *AccI-SmaI* internal fragment of *hrpN*$_{Ecc}$ was used as the probe.

ACKNOWLEDGMENTS

This research was supported by the National Science Foundation (grant DMB-94-19403) and the Food for the 21st Century Program of the University of Missouri. This article is journal series 12,459 of the Missouri Agricultural experiment station. We thank Alan Collmer for the plasmid carrying the *hrpN* DNA of *E. chrysanthemi*, A. J. Novacky and S. Pike for assistance with assays for the HR, and J. E. Schoelz for reviewing the manuscript.

LITERATURE CITED

Bainton, N. J., Bycroft, B. W., Chhabra, S. R., Stead, P., Gledhill, L., Hill, P. J., Rees, C. E. D., Winson, M. K., Salmond, G. P. C., Stewart, G. S. A. B., and Williams, P. 1992. A general role for the *lux* autoinducer in bacterial cell signalling: control of antibiotic biosynthesis in *Erwinia*. Gene 116:87-91.

Barras, F., Thurn, K. K., and Chatterjee, A. K. 1987. Resolution of four pectate lyase structural genes of *Erwinia chrysanthemi* (EC16) and characterization of the enzymes produced in *Escherichia coli*. Mol. Gen. Genet. 209:319-325.

Bauer, D. W., Bogdanove, A. J., Beer, S. V., and Collmer, A. 1994. *Erwinia chrysanthemi hrp* genes and their involvement in soft rot pathogenesis and elicitation of the hypersensitive response. Mol. Plant-Microbe Interact. 7:573-581.

Bauer, D. W., Wei, Z.-M., Beer, S. V., and Collmer, A. 1995. *Erwinia chrysanthemi* harpin$_{Ech}$: An elicitor of the hypersensitive response that contributes to soft-rot pathogenesis. Mol. Plant-Microbe Interact. 8:484-491.

Castilho, B. A., Olfson, P., and Casadaban, M. J. 1984. Plasmid insertion mutagenesis and *lac* gene fusion with mini-Mu bacteriophage transposons. J. Bacteriol. 158:488-495.

Chatterjee, A. K. 1980. Acceptance by *Erwinia* spp. of R plasmid R68.45 and its ability to mobilize the chromosome of *Erwinia chrysanthemi*. J. Bacteriol. 142:111-119.

Chatterjee, A., Cui, Y., Liu, Y., Dumenyo, C. K., and Chatterjee, A. K. 1995. Inactivation of *rsmA* leads to overproduction of extracellular pectinases, cellulases, and proteases in *Erwinia carotovora* subsp. *carotovora* in the absence of the starvation /cell density sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone. Appl. Environ. Microbiol. 61:1959-1967.

Chatterjee, A., McEvoy, J. L., Chambost, J. P., Blasco, F., and Chatterjee, A. K. 1991. Nucleotide sequence and molecular characterization of *pnlA*, the structural gene for damage-inducible pectin lyase of *Erwinia carotovora* subsp. *carotovora 71*. J. Bacteriol. 173:1765-1769.

Cui, Y., Chatterjee, A., Liu, Y., Dumenyo, C. K., and Chatterjee, A. K. 1995. Identification of a global repressor gene, *rsmA*, of *Erwinia carotovora* subsp. *carotovora* that controls extracellular enzymes, N-(3-oxohexanoyl)-L-homoserine lactone, and pathogenicity in soft-rotting *Erwinia* spp. J. Bacteriol. 177: 5108-5115.

Davis, K. R., and Ausubel, F. M. 1989. Characterization of elicitor-induced defense responses in suspension-cultured cells of *Arabidopsis*. Mol. Plant-Microbe Interact. 2:363-368.

Davis, K. R., Lyon, G. D., Darvill, A. G., and Albersheim, P. 1984. Host-Pathogen Interactions. XXV. Endopolygalacturonic acid lyase from *Erwinia carotovora* elicits phytoalexin accumulation by releasing plant cell wall fragments. Plant Physiol. 74:52-60.

Figurski, D. H., and Helinski, D. R. 1979. Replication of an origin-containing derivative of a plasmid RK2 depend on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA 76:1648-1652.

Fuqua, W. C., Winans, S. C., and Greenberg, E. P. 1994. Quorum sensing in bacteria: The LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. 176:269-275.

Goodman, R. N., and Novacky, A. J. 1994. The Hypersensitive Reaction in Plants to Pathogens: A Resistance Phenomenon. American Phytopathological Society, St. Paul, MN.

Gray, P. M., and Greenberg, E. P. 1992. Physical and functional maps of the luminescence gene cluster in an autoinducer deficient *Vibrio fischeri* strain isolated from a squid light organ. J. Bacteriol. 174: 4384-4390.

Keen, N. T. 1992. The molecular biology of disease resistance. Plant Mol. Biol. 19:109-122.

Keen, N. T., Tamaki, S., Kobayashi, D., and Trollinger, D. 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene 70:191-197.

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44: 301-307.

Jones, S., Yu, B., Bainton, N. J., Birdsall, M., Bycroft, B. W., Chhabra, S. R., Cox, A. J. R., Golby, P., Reeves, P. J., Stephens, S., Winson, M. K., Salmond, G. P. C., Stewart, G. S. A. B., and Williams, P. 1993. The *lux* autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora and Pseudomonas aeruginosa*. EMBO J. 12:2477-2482

Laby, R. J., and Beer, S. V. 1992. Hybridization and functional complementation of the *hrp* gene cluster from *Erwinia amylovora* strain Ea321 with DNA of other bacteria. Mol. Plant-Microbe Interact. 5: 412-419.

Lerner, C. G., and Inouye, M. 1990. Low copy number plasmids for regulated low-level expression of cloned genes in *Escherichia coli* with blue/white insert screening capability. Nucleic Acids Res. 18:4631.

Liu, Y., Murata H., Chatterjee, A., and Chatterjee, A. K. 1993. Characterization of a novel regulatory gene *aepA* that controls extracellular enzyme production in the phytopathogenic bacterium *Erwinia carotovora* subsp. *carotovora*. Mol. Plant-Microbe Interact. 6:299-308.

Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mukherjee, A., Cui, Y., Liu, Y., Dumenyo, C. K., and Chatterjee, A. K. 1996a. Global regulation in *Erwinia* species by *Erwinia carotovora rsmA*, a homologue of *Escherchia coli csrA:* Repression of secondary metabolites, pathogenicity and hypersensitive reaction. Microbiology 142:427-434.

Mukherjee, A., Cui, Y., Liu, Y., Dumenyo, C. K., and Chatterjee, A. K. 1996b. A global regulatory gene controls secondary metabolites, motility, and pathogenicity factors in *Erwinia amylovora*. Acta Hortic. 411:237-241.

Murata, H., McEvoy, J. L., Chatterjee, A., Collmer, A., and Chatterjee, A. K. 1991. Molecular cloning of an *aepA* gene that activates production of extracellular pectolytic, cellulolytic, and proteolytic enzymes in *Erwinia carotovora* subsp. *carotovora*. Mol. Plant-Microbe Interact. 4:239-246.

Novacky, A., Acedo, G., and Goodman, R. N. 1973. Prevention of bacterially induced hypersensitive reaction by living bacteria. Physiol. Plant Pathol. 3:133-136.

Palva, T. K., Holmstrom, K.-O., Heino, P., and Palva, E. T. 1993. Induction of plant defense response by exoenzymes of *Erwinia carotovora* subsp. *carotovora*. Mol. Plant-Microbe Interact. 6:190-196.

Pearson, J. P., Gray, K. M., Passador, L., Tucker, K. D., Eberhard, A., Iglewski, B. H., and Greenberg, E. P. 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc. Natl. Acad. Sci. USA 91:197-201

Pirhonen, M., Flego, D., Heikinheimo, R., and Palva, E. T. 1993. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen, *Erwinia carotovora*. EMBO J. 12:2467-2476.

Salmond, G. P. C., Bycroft, B. W., Stewart, G. S. A. B., and Williams, P. 1995. The bacterial "enigma": Cracking the code of cell-cell communication. Mol. Microbiol. 16:615-624.

Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Swift, S., Bainton, N. J., and Winson, M. K. 1994. Gram-negative bacterial communication by N-acyl homoserine lactones: A universal language? Trends Microbiol. 2:193-198.

Wei, Z. M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A., and Beer, S. V. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science 257:85-88.

Zink, R. T., Kemble, R. J., and Chatterjee, A. K. 1984. Transposon Tn*5* mutagenesis in *Erwinia carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica*. J. Bacteriol. 157:809-814.

For the 8th International Congress on Molecular Plant-Microbe Interactions; July 14-19, 1996; Knoxvill , TN:

HARPIN IS NOT NECESSARY FOR THE PATHOGENICITY OF *ERWINIA STEWARTII* ON MAIZE.

Musharaf Ahmad, D. R. Majerczak, and D. L. Coplin*. D pt. of Plant Pathology, The Ohio State University, Columbus, OH 43210-1087, USA.

Erwinia stewartii elicits a hypersensitive response (HR) in tobacco if expression of the *hrp*-like *wts* regulon is enhanced. A clone containing *E. amylovora hrpN*$_{Ea}$ was used as a hybridization probe to locate a gene for harpin production, *hrpN*$_{Es}$, within the *wts* gene cluster. Transposon mutagenesis and complementation analysis revealed that *hrpN*$_{Es}$ is a monocistronic operon. Sequence analysis indicated that it encodes a 382-amino acid, glycine-rich polypeptide, which lacks cysteine and an N-terminal signal peptide. Harpin$_{Es}$ is 58% identical and 78% homologous to harpin$_{Ea}$, and 41% identical and 66% homologous to harpin$_{Ech}$ from *E. chrysanthemi*. Purified harpin$_{Es}$ was protease sensitive and heat-stable, and it elicited a typical HR in tobacco leaves. Antibodies to harpin$_{Es}$ cross-reacted with harpin$_{Ea}$, and conversely. Harpin$_{Es}$ was found in cytoplasmic, membrane, and extracellular fractions. Chromosomal mutations in *hrpN*$_{Es}$ were constructed by Tn*5* mutagenesis and marker-exchange. The mutants were HR- and did not produce detectable harpin in Western blots. However, they remained fully pathogenic on maize seedlings with respect to symptom severity, ED_{50} and response time, and they grew as well as the wild-type strain *in planta*. Likewise, loss of harpin did not affect the ability of a *hrpN*$_{Es}$ mutant to grow endophytically in several grasses. *wtsB*, *wtsD*, and *wtsF* mutants accumulated Harpin$_{Es}$ intracellularly, indicating that thes DNA regions ar necessary for harpin secretion.

MPMI Vol. 8, No.5, 1995, pp. 717–732. © 1995 The American Phytopathological Society

The HrpZ Proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea*, and *tomato* Are Encoded by an Operon Containing *Yersinia ysc* Homologs and Elicit the Hypersensitive Response in Tomato but not Soybean

Gail Preston,[1] Hsiou-Chen Huang,[2] Sheng Yang He,[3] and Alan Collmer[1]

[1]Department of Plant Pathology, Cornell University, Ithaca, NY 14853 U.S.A.; [2]Agricultural Biotechnology Laboratories, National Chung-Hsing University, Taichung, Taiwan 40227 R.O.C.; [3]Department of Plant Pathology, University of Kentucky, Lexington, KY 40546 U.S.A.

Received 6 February 1995. Accepted 18 May 1995.

The *Pseudomonas syringae* pathovars are composed of host-specific plant pathogens that characteristically elicit the defense-associated hypersensitive response (HR) in n nhost plants. *P. s.* pv. *syringae* 61 secretes an HR elicitor, harpin$_{Pss}$ (HrpZ$_{Pss}$), in a *hrp*-dependent manner. An internal fragment of the *P. s.* pv. *syringae* 61 *hrpZ* gene was used to clone the *hrpZ* locus from *P. s.* pv. *glycinea* race 4 (bacterial blight of soybean) and *P. s.* pv. *tomato* DC3000 (bacterial speck of tomato). DNA sequence analysis revealed that *hrpZ* is the second ORF in a polycistronic operon. The amino acid sequence identities of HrpZ$_{Pss}$/HrpZ$_{Psg}$ and HrpZ$_{Pss}$/HrpZ$_{Pst}$ were 79 and 63%, respectively. Although none of the HrpZ proteins showed significant overall sequence similarity with other known proteins, HrpZ$_{Pst}$ contained a 24-amino acid sequence that is homologous with a region of the PopA1 elicitor protein of the tomato pathogen, *Pseudomonas solanacearum* GMI1000. *hrpA*, the upstream ORF, was highly divergent: The amino acid sequence identities of HrpA$_{Pss}$/HrpA$_{Psg}$ and HrpA$_{Pss}$/HrpA$_{Pst}$ were 91 and 28%, respectively, and n HrpA sequence showed similarity to known proteins. In contrast, the predicted products of the downstream ORFs in *P. s.* pv. *syringae* and *P. s.* pv. *tomato*, *hrpB*, *hrpC*, *hrpD*, and *hrpE* showed varying levels of similarity to those of *yscI*, *yscJ*, *yscK*, and *yscL*. These are colinearly arranged genes in the *virC* locus of *Yersinia* spp., which are involved in the secretion of the Yop virulence proteins via the type III pathway. The similarity of the Ysc proteins was generally stronger in comparisons with the *P. s.* pv. *tomato* Hrp proteins. The HrpZ proteins were purified by heat denaturation of contaminating proteins followed by ammonium sulfate fractionation, hydrophobic chromatography, and gel electrophoresis. All three HrpZ proteins elicited the HR in tomato, whereas none of them elicited significant necrosis in soybean. The results indicate that HrpZ is enc ded in an operon containing some of the genes involved in its own secretion and suggest that HrpZ structure does not directly determine bacterial host range.

Corresponding author: A. Collmer; E-mail arc2@cornell.edu

Phytopathogenic strains of *Pseudomonas syringae* cause two patterns of necrosis when the bacteria invade a plant. On a susceptible ("compatible") host, a necrotic lesion often develops over a period of days, with necrosis spreading as the bacteria multiply and the plant becomes diseased. On a resistant or nonhost plant, a localized cellular necrosis is induced within 24 to 48 h, and bacterial multiplication is inhibited. This was first reported by Klement (1963; Klement et al. 1964), who observed that when high concentrations of phytopathogenic bacteria are infiltrated into an incompatible plant they elicit a visible necrosis which is limited to the infiltrated area. This reaction, called the hypersensitive response (HR), involves localized cell death and production of anti-microbial compounds at the site of pathogen invasion (Bonas 1994). The ability of *P. syringae* and other nontumorigenic, gram-negative, bacterial pathogens to elicit the HR is governed by *hrp* genes. Typical Hrp$^-$ mutants are pleiotropically defective in planta: They do not elicit the HR in nonhosts and they fail to multiply and cause disease in host plants (Lindgren et al. 1986). Clusters of *hrp* genes have been identified in many gram-negative phytopathogenic bacteria (Bonas 1994). A 25-kb *hrp* cluster from *P. s.* pv. *syringae* 61 is sufficient to confer the tobacco HR phenotype, but not the pathogenic phenotype on nonpathogenic bacteria (Huang et al. 1988). *hrp* genes have also been cloned and characterized extensively from *P. s.* pv. *phaseolicola* NPS3121, *P. solanacearum* GM1000, *Xanthomonas campestris* pv. *vesicatoria* 75-3, and *Erwinia amylovora* Ea321 (Lindgren et al. 1986; Boucher et al. 1987; Beer et al. 1991; Bonas et al. 1991). Certain *hrp* genes are widely conserved among these pathogens, and several encode components of a protein secretion pathway that is similar to the type III pathway used by *Yersinia*, *Shigella*, and *Salmonella* spp. to secrete extracellular proteins involved in animal pathogenesis (Van Gijsegem et al. 1993). One activity of the *hrp*-encoded secretion pathway in phytopathogenic bacteria is the secretion of proteinaceous elicitors of the HR, which are also encoded by *hrp* genes.

The first *hrp*-encoded elicitor characterized was harpin$_{Ea}$ from *E. amylovora* (Wei et al. 1992). Similar elicitors have since been isolated from other bacteria, including *P. s.* pv. *syringae* 61, *P. solanacearum* GMI1000, and *E. chrysanthemi*

EC16 (He et al. 1993; Arlat et al. 1994; Bauer et al. 1994). Proteins in this family of elicitors share several general characteristics. They are glycine rich, heat-stable, lack cysteine, and appear highly susceptible to proteolysis. They lack an N-terminal signal peptide, but they are secreted to the bacterial milieu. Their expression and secretion is dependent on *hrp* genes. The biological role of these proteins in pathogenesis has not yet been determined, but the purified proteins can induce an HR on a nonhost plant such as tobacco. However, there are significant differences in the organization of the elicitor operons and the activity of the elicitors, which suggests that the *Erwinia* harpins, the *P. syringae hrpZ* product and the *P. solanacearum popA* product may represent three distinct classes of elicitors. In this work we will refer to the *P. s.* pv. *syringae* elicitor as $HrpZ_{Pss}$ rather than $harpin_{Pss}$ (He et al. 1993). This distinction is supported by the weak similarity of the amino acid sequences of the four proteins, with the only xception being the C-terminal halves of the *Erwinia* harpins (Bauer et al. 1994).

The location of known elicitor genes in reference to the *hrp* cluster varies in *P. s.* pv. *syringae*, *P. solanacearum*, and *E. amylovora*. *hrpN* and *hrpZ* are contiguous or within the *hrp* cluster, whereas *popA* lies outside (although near) the *P. solanacearum hrp* cluster (Wei et al. 1992; He et al. 1993; Arlat et al. 1994). There are no genes downstream of the elicitor gene in either the *hrpN* or the *popA* operons, which means that mutations in the elicitor genes do not have a polar effect on the Hrp phenotype, and mutant construction is straightforward. In contrast, mutagenesis and complementation studies of the *hrp* cluster from *P. s.* pv. *syringae* 61 have indicated that *hrpZ* lies upstream of at least one other *hrp* gene within an operon (Huang et al. 1991; Xiao et al. 1992).

In *E. amylovora* and *E. chrysanthemi*, harpins have been demonstrated to be sufficient and necessary to elicit the HR, and mutation of *hrpN* in *E. amylovora* has shown that $harpin_{Ea}$ is required for pathogenesis (Wei et al. 1992). However *hrpN* mutants of *E. chrysanthemi* can establish infections, albeit at a significantly reduced frequency, which suggests that $harpin_{Ech}$ is important but not essential for pathogenesis (Bauer et al. 1995). In contrast, a *popA* mutant of *P. solanacearum* is fully pathogenic on susceptible hosts, indicating that PopA1 is not required for pathogenesis (Arlat et al. 1994).

These elicitors may play a role in controlling the host specificity exhibited by *E. amylovora* and plant pathogenic pseudomonads such as *P. syringae* and *P. solanacearum*. However it is difficult to compare the activity of $HrpZ_{Pss}$ and $harpin_{Ea}$ in host and nonhost plants because legumes and rosaceous plants, the hosts of *P. s.* pv. *syringae* 61 and *E. amylovora* Ea321, respectively, respond poorly to preparations of any of these elicitor proteins (Wei et al. 1992; He et al. 1993). PopA1 from *P. solanacearum* does appear to act in a host-specific manner, inducing an HR on resistant lines of petunia and the nonhost tobacco, but not on susceptible lines of petunia or tomato (Arlat et al. 1994). This phenotype is similar to that of *avr* genes, but PopA1 is distinct from known Avr proteins in eliciting the HR directly on resistant plants. $Harpin_{Ech}$ elicits an HR on some c mpatible hosts of *E. chrysanthemi*, but in contrast to the other three bacteria *E. chrysanthemi* is a broad-host range pathogen and the activity of $harpin_{Ech}$ may not be representative of elicitor activity in a highly host-specific system (Bauer t al. 1995).

In previous work we cloned and characterized the *hrpZ* gene from *P. s.* pv. *syringae* 61, a weak pathogen of bean, and demonstrated with Southern and immunoblots that ther pathovars of *P. syringae* contain homologs of this gene (He et al. 1993). This supported the hypothesis that HrpZ represents a family of elicitors common to all pathogenic strains f *P. syringae*. We report here the isolation of homologs of $HrpZ_{Pss}$ from two other experimentally important pathovars of *P. syringae*-*P. s.* pv. *tomato* and *P. s.* pv. *glycinea*. Examining HrpZ from these three pathovars enabled us to look within this family of elicitors for variations in sequence and activity which could indicate a role in host range determination. In addition, we characterized the two genes flanking *hrpZ* in *P. s.* pv. *syringae* and *P. s.* pv. *glycinea* and the entire *hrpZ* operon of *P. s.* pv. *tomato*. In conjunction with an accompanying paper (Huang et al. 1995), this completes the sequence of the *P. s.* pv. *syringae* 61 *hrp* genes carried on pHIR11 and provides clues to the function of the genes downstream of *hrpZ*. A preliminary account of portions of this work has been published (Collmer et al. 1994).

RESULTS

Cloning *hrpZ* from *P. s.* pv. *tomato* and *P. s.* pv. *glycinea*.

We previously used Southern hybridization to demonstrate that both *P. s.* pv. *glycinea* race 4 and *P. s.* pv *tomato* DC3000 contain sequences homologous to a 0.75 kb *Bst*XI internal fragment of *hrpZ* from *P. s.* pv. *syringae* (He et al. 1993). The same probe was used to screen genomic libraries of *P. s.* pv. *glycinea* and *P. s.* pv. *tomato*. The libraries were constructed in *E. coli* DH5α by inserting 8- to 12-kb fragments from partial *Sau*3AI digests of genomic DNA into the *Bam*HI site of pUCP19. The screen identified two plasmids with inserts of approximately 10 kb: pCPP2201 (*P. s.* pv. *tomato*) and pCPP2200 (*P. s.* pv. *glycinea*). The same *Bst*XI fragment was used to probe a Southern blot of pCPP2201 and pCPP2200 digested with *Bam*HI, *Eco*RI, and *Pst*I. The probe identified two *Pst*I fragments of 2.2 and 2.4 kb from pCPP2201 and pCPP2200 respectively (Fig. 1). The two *Pst*I fragments were cloned into the *Pst*I site of pBluescript II SK(–) (Stratagene, La Jolla, CA) in *E. coli* DH5α to create the plasmids pCPP2202 to pCPP2205, with the inserts in both orientations with respect to the *lac* promoter. Cell lysates of *E. coli* DH5α containing pCPP2203 ($hrpZ_{Pst}$ in the vector promoter orientation) and pCPP2202 ($hrpZ_{Psg}$ in the vector promoter orientation) induced an HR on tobacco, but those from cells containing pCPP2205 ($hrpZ_{Pst}$ in the opposite orientation of the vector promoter) and pCPP2204 ($hrpZ_{Psg}$ in the opposite orientation of the vector promoter) did not. HR activity was retained after incubating the lysate for 10 min at 100°C and removing denatured proteins by centrifugation. Insensitivity to heat treatment is a characteristic feature f previously isolated HR elicitors. Proteins in the lysates were separated on an SDS-polyacrylamide gel, transferred to an Immobilon-P membrane and immunoblotted with antibodies raised against purified $HrpZ_{Pss}$. Cross-reacting proteins of a similar size to $HrpZ_{Pss}$ were observed and provisionally named $HrpZ_{Psg}$ and $HrpZ_{Pst}$ (Fig. 2, lanes 2 and 4).

The intensity of the $HrpZ_{Psg}$ and $HrpZ_{Pst}$ bands was quite low in comparison to the band for $HrpZ_{Pss}$ expressed from pSYH10 in *E. coli* DH5α (Fig. 2, lane 1). This impli d either

that expression was low due to the distance of the cloned gene from the *lac* promoter or that HrpZ_{Psg} and HrpZ_{Pst} did not hybridize strongly to the antibodies. A band corresponding to HrpZ_{Pss} from pSYH10 could be clearly seen on a Coomassie-stained gel, but the bands for HrpZ_{Psg} and HrpZ_{Pst} were indistinct, which implies that low expression was a primary reason for the low signal. In an attempt to improve the level of expression of HrpZ_{Psg} and HrpZ_{Pst} we subcloned *Eco*RI-*Bam*HI fragments containing the inserts from pCPP2202 and pCPP2203 behind the T7 promoter of pET21(+) in *E. coli* BL21(DE3) to create the plasmids pCPP2206 and pCPP2207. The T7 promoter enabled a moderate improvement in protein expression (Fig. 2, lanes 3 and 5).

A common arrangement of ORFs in the *hrpZ* operons of *P. s.* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato* revealed by DNA sequence analysis.

Previously, we determined the complete nucleotide sequence of *hrpZ* from *P. s.* pv. *syringae* by sequencing a 1.4-kb subclone of pHIR11 (a cosmid containing the entire *hrp* cluster from *P. s.* pv. *syringae*) (He et al. 1993). In addition, analysis of the complementation groups and transcriptional

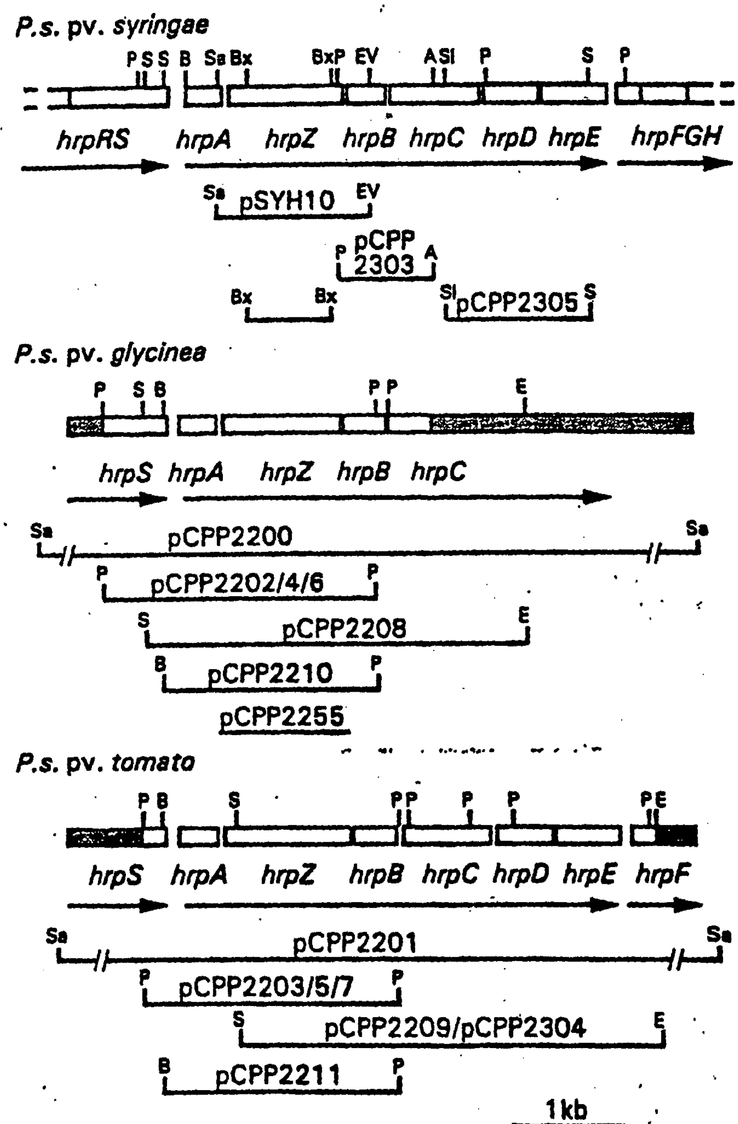


Fig. 1. Physical maps of the *hrpZ* regions from *Pseudomonas syringae* pv. *syringae* 61, *P. s.* pv. *glycinea* race 4, and *P. s.* pv. *tomato* DC3000 and clones used in this study. Open boxes represent sequenced ORFs; filled boxes represent unsequenced DNA. Direction of transcription is indicated by the arrows. Key restriction sites within the sequenced regions are indicated, along with the subclones used in this study. The 0.75-kb *Bst*XI fragment from $hrpZ_{Pss}$ used as a probe for *hrpZ* genes in other pathovars is also shown. Restriction endonuclease abbreviations: A, *Age*I*; B, *Bgl*II; Bx, *Bst*XI*; E, *Eco*RI; EV, *Eco*RV*; P, *Pst*I; S, *Sac*I; Sa, *Sau*3A*; Sl, *Sal*I*. * Not all sites are shown.

units of pHIR11 using Tn*phoA* and Tn5-*gus*A1 mutagenesis (Huang et al. 1991; Xiao et al. 1992) suggested that *hrpZ* lay within an operon, upstream of at least one other *hrp* gene. Further subclones of pHIR11 were used to determine the sequence of the entire *hrpZ*$_{Pss}$ operon (this study, Huang et al. 1995). We also determined the sequence of (i) the 2.2- and 2.4-kb *Pst*I subclones from pCPP2201 (*hrpZ*$_{Pst}$*) and pCPP2200 (*hrpZ*$_{Psg}$*), (ii) an overlapping 3.7-kb *Sac*I-*Eco*RI subclone from pCPP2201 (designated pCPP2209), and (iii) part of an overlapping 3.6-kb subclone from pCPP2200 (designated pCPP2208), as shown in Figure 1. This yielded the sequence of the entire *P. s.* pv. *tomato hrpZ* operon and the first half of the *P. s.* pv. *glycinea* operon. The sequenced region of *P. s.* pv. *syringae* and *P. s.* pv. *tomato* extends from *hrpS* (Xiao et al. 1994), through the *hrpZ* operon to the beginning of the *hrpH* operon (Huang et al. 1992), demonstrating that the organization of this region of the *hrp* cluster is conserved in both pathovars.

Codon preference analysis of the DNA sequence, using *P. s.* pv. *syringae* codon usage data, predicted that *hrpZ* was the second of six ORFs, all oriented in the same direction, an arrangement conserved in *P. s.* pv. *tomato* and at least the first four ORFs of *P. s.* pv. *glycinea*. The sequence of the noncoding DNA is shown in Figure 3. Five of the six ORFs have clear potential ribosome binding sites. The fifth ORF has a putative ribosome binding site in *P. s.* pv. *syringae*, but the site in *P. s.* pv. *tomato* is less clear, the initiation codon shown being selected by alignment with the ORF in *P. s.* pv. *syringae*. In the absence of recognizable terminator elements downstream of the first five ORFs it seems likely that the six ORFs represent a single operon, transcribed from upstream of the first ORF. The five predicted ORFs were provisionally named *hrpA* through *hrpE*, as shown in Figures 1 and 3.

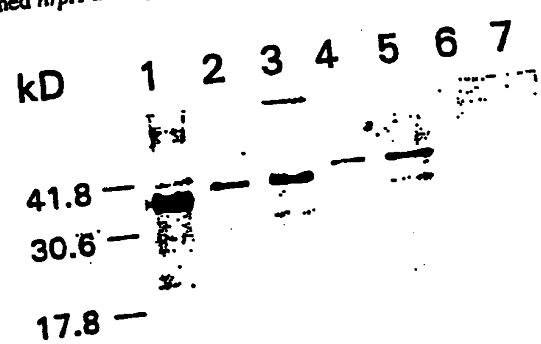


Fig. 2. Immunoblot showing expression of cloned *hrpZ* in *E. coli*. Cultures were grown in LM to an OD_{600} of 0.8 to 1.0 at 30°C, collected by centrifugation and resuspended in 5 mM MES, pH 5.5. For lanes 3, 5 and 7, and 4, T7 expression was induced with 1 mM IPTG when the cells reached an OD_{600} of 0.6, 3 h prior to collection. The cells were disrupted by sonication, and the crude lysate was partially purified by removal of the insoluble fraction after incubating the samples at 100°C for 10 min. SDS-loading buffer was added and the samples were incubated at 100°C for 2 min. The proteins were resolved by SDS-polyacrylamide gel electrophoresis. Following electrophoresis the proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA), probed with anti-HrpZ$_{Pss}$ antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase. Lanes: 1, *E. coli* DH5α (pSYH10) (HrpZ$_{Pss}$); 2, *E. coli* DH5α (pCPP2202)(HrpZ$_{Psg}$) 3, *E. coli* BL21(DE3) (pCPP2206)(HrpZ$_{Psg}$); 4, *E. coli* DH5α (pCPP2203)(HrpZ$_{Pst}$); 5, *E. coli* BL21(DE3) (pCPP2207)(HrpZ$_{Pst}$); 6, *E. coli* DH5α (pBluescript II); 7, *E. coli* BL21(DE3)(pET21+).

A *hrp/avr* promoter consensus sequence lies upstream of the *hrpZ* operons of the three *P. syringae* pathovars.

The conserved sequence GGAACC—16bp—CCACNNA lies 50 bp upstream of the initiation codon of *hrpA* in all three pathovars (Fig. 3). This motif has been identified in the promoter regions of many *avr* and *hrp* genes (Innes et al. 1993; Shen and Keen 1993), and appears to be involved in positive regulation by HrpL, a putative alternative sigma factor which is itself positively regulated by HrpR and HrpS (Xiao and Hutcheson 1994). HrpL is a member of a family of alternative sigma factors, many of which are involved in secretion of extracellular factors in response to environmental stimuli (Lonetto et al. 1992). The presence of this promoter motif further supports the suggestion that the six ORFs form a single transcriptional unit which is regulated in a *hrp*-dependent manner. This motif can also be found beyond *hrpE*, upstream of *hrpFGH* in *P. s.* pv. *syringae* and *P. s.* pv. *tomato*, as indicated at the bottom of Figure 3, suggesting that the latter three ORFs form an independent *hrp*-regulated transcriptional unit in these two pathovars.

Comparison of the HrpZ proteins of the three *P. syringae* pathovars.

The predicted amino acid sequences for HrpZ from each of the three pathovars are aligned in Figure 4. Although the proteins migrate slightly anomalously on an SDS polyacrylamide gel, the relative sizes of the estimated molecular weights correspond to the predicted values, with HrpZ$_{Pss}$ being the largest of the three proteins (36.5 kDa), followed by HrpZ$_{Psg}$ (35.3 kDa) and HrpZ$_{Pst}$ (34.7 kDa). Amino-terminal sequencing of the first 10 to 15 residues of purified HrpZ$_{Psg}$ and HrpZ$_{Pst}$ confirmed the predicted initiation codons of both proteins, which aligned with the start codon of HrpZ$_{Pss}$ as shown in Figures 3 and 4. The proteins expressed in *E. coli* appear to be the same size as those recovered from the supernatants of *P. s.* pv. *glycinea* and *P. s.* pv. *tomato*, indicating that the cloned gene is intact and that there are no large posttranslational modifications or deletions of HrpZ taking place in *P. syringae* but not in *E. coli*.

The amino acid sequence of HrpZ$_{Psg}$ is quite highly conserved with respect to HrpZ$_{Pss}$, having 87% similarity and 79% identity. HrpZ$_{Pst}$ is less conserved with respect to the two other proteins, with 75% similarity and 63% identity to HrpZ$_{Pss}$. However, the physical features of HrpZ$_{Psg}$ and HrpZ$_{Pst}$ are almost identical to those reported for HrpZ$_{Pss}$ (He et al. 1993). All three are glycine-rich proteins lacking cysteine and tyrosine. HrpZ$_{Pst}$ is the most glycine rich, being 15.7% glycine. The proteins lack the hydrophobic signal sequence used to target proteins for secretion via the Sec export pathway (Pugsley 1989). Analysis of the amino acid sequence fails to identify any obviously significant secondary structure, which is consistent with their sensitivity to proteases, and supports the suggestion that they adopt a fairly open structure in aqueous solution.

In our previous analysis of HrpZ$_{Pss}$ (He et al. 1993), we noted the presence of two sets of short, direct repeats. Only one of these repeats, GGGLGTP, is conserved in the three proteins, with the substitution of a serine for threonine in the first repeat of both HrpZ$_{Psg}$ and HrpZ$_{Pst}$. The significance of these repeats, if any, is unknown. A database search with each of the three proteins using the BLAST algorithm (Altschul et

```
syringae  TTTTTTGCAG AAGATCTGGA ACCGATTCGC GGACACATGC CACCTAGCTG
glycinea  TTTTTTGCA. GAGCGCTGGA ACCGATTTAA GGGTCGTTAC CACTA.TCTG
  tomato  TTTTTTGCAA AGACGCTGGA ACCGTATCGC AGGCTGCTGC CACTAGTGAG

syringae  TACCAAGCAA TTACGCTGGT ACAGACGAAG GGGTATGACG TTATG-----
glycinea  TACCAAGCAA TTACGCTGGT ACAGACCAAG GGGTATCACG TTATG-----
  tomato  TACCAAGCAA TCACGCTGGT AAATCTTAAG GGGCATCAAA TCATG-----

syringae  ---------- --321bp--- ---------T GATTTCTTG. ACGCCCCTTC
glycinea  ---hrpA--- --321bp--- ---------T GATTTCTTGA ATGCCCCCAT
  tomato  ---------- --336bp--- ---------T AATTATTTCT GATTGCCCCC

syringae  ATACCTGAGG GGGCTGCTAC TTTTAGGAGG TTGTG..ATG ----------
glycinea  CACACAGAGG GGGCTGCTAC TTTGAGGAGG TTGTG..ATG ----------
  tomato  TCATCAGAGG GGGCCGCTAC CTTGGGATGG GCGTTTTATG ----------
          =>    <=== ====
syringae  ---------- --1020bp-- ---------- ---------- ----------
glycinea  ---hrpZ--- --1032bp-- ---------- ---------- ----------
  tomato  ---------- --1107bp-- ---------- ---------- ----------

syringae  ---------- ---------- ---------- ---------- ----------
glycinea  ---------- ---------- ---------- ---------- ----------
  tomato  ---------- ---------- ---------- ---------- ----------

syringae  TGACCGACAA CCGCCTGACG GAGAACTCAC GTG------- ----------
glycinea  TGACTGATAC CCGCCTGACG GAGAACTCAC GTG------- ---hrpB---
  tomato  TGACTGACAG CCGCCTGACG GAGAACCAGT GTG------- ----------

syringae  --369bp--- ---------- TAGAGGTTTC CGTG------ ----------
glycinea  --369bp--- ---------- TAGAGGTTCT CGTG------ ----------
  tomato  --369bp--- ---------- TAGAGGTTTC CGTG------ ----------

syringae  ---------- --801bp--- ---------- ---------- ----------
glycinea  ---hrpC--- incomplete
  tomato  ---------- --801bp--- ---------- ---------- ----------

syringae  ---------- -----TGATG GACCTGACCG CCGAGGACTA TTGGACTCAG
  tomato  ---------- ----ATGATG AGCCTTTCTG CCGAGGATCA CTGGATTCAC

syringae  TGGTGGTGCA ATCCCTGGCC ATGGGCGCAT CCGGGCTGGC AAAGCCGGTT
  tomato  TGGTGGTGCA ACCCCTGGCA GTGGGCACAT TCGGAGTGGC ATGACCGATT

syringae  CGCCGAGCGC TGCGGACTGA CCGTCAGCGA ATGTGAAGCC CTTATG----
  tomato  CGCCAACGCT CGTGGGTTAT CGGTCAGTGA CTGCGATGCG CTCATG----

syringae  ---------- ---hrpD--- --396bp--- ---------- ----------
  tomato  ---------- ---------- --396bp--- ---------- ----------

syringae  --TGAGTAT. .CCGCTCCTC TCTGCACCAG GAATTCTCCC ATG-------
  tomato  --TGAATCCG AACCAGCTTC TCTGCATCAG GAATACGCCC ATG-------

syringae  ---hrpE--- --576bp--- ---------- ---------- -------TGA
  tomato  ---------- --576bp--- ---------- ---------- -------TGA

syringae  AACAGACT.. .........C TTGCGGCGAA AATGGAACCG CTCCACCTGT
  tomato  TACACACTCT CTGCACTCAC TTGATCGCAT GATGGAACCG CTCGGCGGGT

syringae  TTGCTCCACT CAAGGTTTGA ACCTTTCTGC TGGAGTATCA GGACATG
  tomato  TTGCTCCACT CAAGGTTTGA ACCCTTCTGC TGGAGCACCA GGACATG
```

Fig. 3. Nucleotide sequences of the noncoding regions of the *hrpZ* operon from *Pseudomonas syringae* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato*. The sequences flanking the six ORFs of the *hrpZ* operon were aligned using the PILEUP algorithm (Genetics Computer Group). For *P. s.* pv. *syringae* and *P. s.* pv. *tomato* the sequence extends from immediately downstream of *hrpS* to the end of the operon. For *P. s.* pv. *glycinea* the sequenced region terminates at the beginning of *hrpC*. The proposed initiation and termination codons are highlighted for each ORF. The *hrp/avr* consensus sequences upstream of *hrpA* amd *hrpF* are marked by double lines, with the conserved nucleotides in bold and the putative ribosome binding sites for each ORF underlined. A short inverted repeat upstream of *hrpZ* is also indicated with dashed arrows.

al. 1990) did not find significant homology to any other bacterial proteins, with the exception of a single, glycine rich region found only in HrpZ$_{Pst}$ (Fig. 4). This stretch of 24 amino acids has homology at both the nucleotide and amino acid level to a region of the host-specific elicitor PopA1 from *P. solanacearum*, as shown at the bottom of Figure 4. There is no overall similarity of the amino acid and nucleotide sequences of HrpZ to the HR elicitors characterized from *E. amylovora*, *E. chrysanthemi*, and *P. solanacearum* except to a degree accounted for by their similar composition.

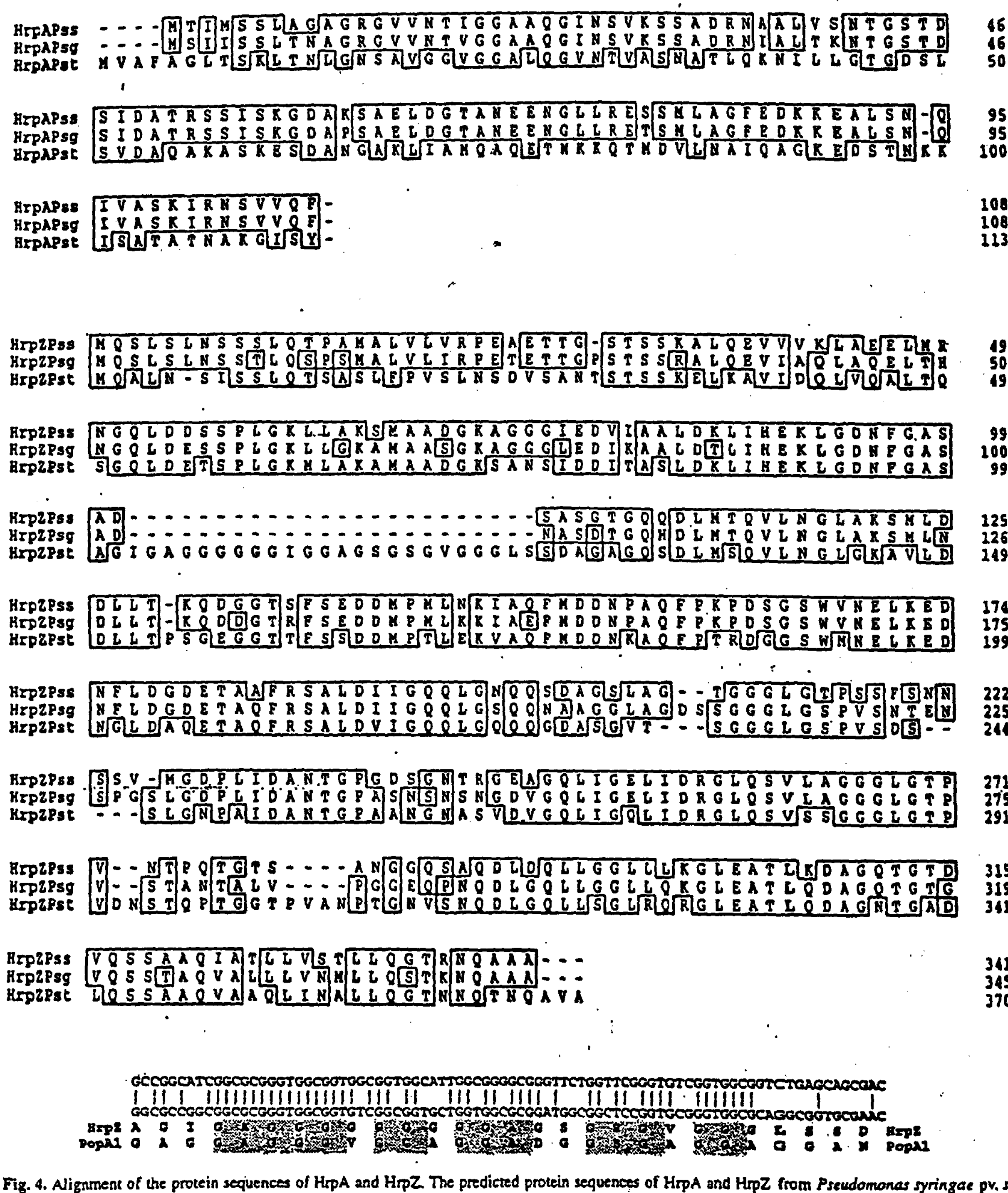


Fig. 4. Alignment of the protein sequences of HrpA and HrpZ. The predicted protein sequences of HrpA and HrpZ from *Pseudomonas syringae* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato* were aligned using the PILEUP algorithm (Genetics Computer Group). The alignment of a unique glycine rich region of HrpZ$_{Pst}$ with a homologous region of PopA1 from *P. solanacearum* is also shown.

The predicted HrpA protein of *P. s.* pv. *tomato* differs substantially from that of *P. s.* pv. *syringae* and *P. s.* pv. *glycinea*.

The first ORF of the *hrpZ* operon starts 50 bp downstream of the conserved *hrp/avr* promoter motif, as shown in Figure 3. The predicted product is a small (11 kDa), hydrophilic protein with a hydrophobic N-terminus. An alignment of the amino acid sequences from all three pathovars is shown in Figure 4. Although the predicted sequences of HrpA from *P. s.* pv. *syringae* and *P. s.* pv. *glycinea* are highly conserved, with 92% similarity and 91% identity to each other, HrpA from *P. s.* pv. *tomato* is quite divergent, having only 42% similarity and 28% identity to HrpA from *P. s.* pv. *syringae*. The presence of a ribosome binding site and the highly conserved character of HrpA in two of the three pathovars supports the hypothesis that HrpA is translated. T7 polymerase-dependent expression of *hrpA* (described below) provides further evidence for production of a HrpA protein. Cell lysates of *E. coli* expressing only HrpA did not elicit the HR on tobacco (data not shown), which suggests that it does not contribute directly to the HR. The role of HrpA in the bacterium is unknown, and it shows no significant homology to any previously characterized proteins.

T7 expression studies.

To confirm the production of proteins corresponding to the two sets of newly cloned *hrpA* and *hrpZ* genes, the *BglII-PstI* fragments from *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* were subcloned into pET21(+) and the products specifically labelled by T7 promoter/polymerase-dependent expression in *E. coli* BL21(DE3) cells incubated with [^{35}S]-methionine (Studier et al. 1990). Radiolabeled proteins in the cell lysate were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 5). Lysates of cells containing pCPP2211 displayed unique bands which corresponded well with the predicted molecular weight of HrpA (11.5 kDa) and were consistent with the previously observed mobility of HrpZ$_{Pst}$ (Fig. 5, lane 2). Lysates of cells containing pCPP2210 contained bands corresponding to HrpZ$_{Psg}$ (36 kDa) and HrpA (11 kDa)(Fig. 5, lane 3). No HrpB band was visible in the products of pCPP2211 (Fig. 5, lane 2), but this could potentially be attributed to the omission of cysteine, which is not required for HrpA and HrpZ synthesis, from the amino-acids added to the reaction mixture. T7 expression of HrpB was independently confirmed for both *P. s.* pv. *syringae* and *P. s.* pv. *tomato* using a 0.84-kb *PstI-AgeI* fragment of pHIR11 and the 3.7-kb *SacI-EcoRI* fragment from pCPP2209, subcloned into LITMUS 28 to construct the plasmids pCPP2303 and pCPP2304. T7 expression in *E. coli* BL21(DE3) cells was performed as outlined above and in Figure 5. In each case a protein of about 13 kDa was observed, which corresponds well with the predicted molecular weight of HrpB from each of the two pathovars (data not shown). In an accompanying study Huang et al. (1995) have confirmed the production of proteins corresponding to HrpC, HrpD, and HrpE from *P. s.* pv. *syringae* 61. The similarities between the three pathovars suggest that the equivalent ORFs in *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* also encode proteins. However when we independently confirmed the production of HrpD from *P. s.* pv. *syringae* 61 using a 1.3-kb *SalI-SacI* subclone from pHIR11 cloned into pT7-6 (pCPP2305) our results suggested the use of an alternative initiation codon to make a larger (21 kDa) HrpD protein (data not shown). In the absence of a strong ribosome binding site at either of the putative initiation codons, the exact size of HrpD remains uncertain.

The four ORFs downstream of *hrpZ* show varying similarities to *Yersinia* Ysc proteins.

The *hrpC*, *hrpD*, and *hrpE* genes downstream of *hrpZ* in *P. s.* pv. *syringae* 61 have been sequenced and the products identified using T7 polymerase-dependent expression (Huang et al. 1995). Two of the predicted proteins, HrpC and HrpE, were shown to be homologous to the proteins YscJ and YscL, respectively, which are encoded in the *virC* operon of *Yersinia enterocolitica* and are involved in the type III secretion pathway (Michiels et al. 1991). Homologs of YscJ have also been found in the *hrp* clusters of several other phytopathogenic bacteria, including *P. solanacearum* and *X. campestris* (Fenselau et al. 1992; Gough et al. 1992). Additional homologs are *Salmonella typhimurium* FliP and *Rhizobium fredii* NolT (Jones et al. 1989; Meinhardt et al. 1993). The same four downstream ORFs are found in *P. s.* pv. *tomato*, and the partial sequence of the operon from *P. s.* pv. *glycinea* confirms the presence of the first two of these ORFs, *hrpB* and *hrpC*, in this pathovar (Fig. 6).

HrpB is fairly conserved in all three pathovars, as shown by the alignment presented in Figure 6. It encodes a small serine-rich protein of approximately 13 kDa. BLAST searches using HrpB from either *P. s.* pv. *syringae* or *P. s.* pv. *glycinea* identified no significant homologies, but a search using HrpB from *P. s.* pv. *tomato* identified similarity to the *Yersinia* protein, YscI. YscI is 115 amino acids long, thus slightly shorter than HrpB (127 amino acids). *yscI* lies immediately upstream of *yscJ* in the *virC* operon, which suggests that the downstream ORFs of the *hrpZ* operon might be colinear with a region of the *virC* operon.

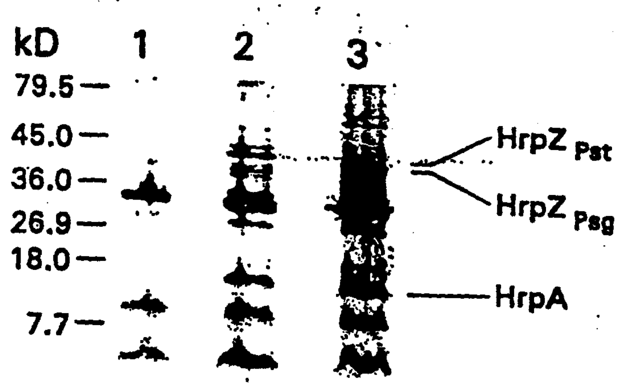


Fig. 5. T7 polymerase-dependent expression and radiolabeling of HrpA and HrpZ. T7 promoter/polymerase expression was carried out using the pET21(+) vector system in *E. coli* BL21(DE3). Cells were grown in LM to an OD$_{600}$ of 0.5, then centrifuged and resuspended in M9 minimal medium supplemented with 0.01% amino acids (lacking methionine and cysteine), glucose and thiamine. Cells were incubated at 30°C for 3 h and then induced with 1 mM IPTG for 10 min, followed by incubation with rifampicin at 300 µg/ml for 30 min. Cells were incubated with 10 µCi [^{35}S]-methionine for 10 min, lysed in SDS-loading buffer, and the proteins were separated by SDS-polyacrylamide electrophoresis and visualized by autoradiography. *E. coli* BL21(DE3) cells carried the following plasmids in lanes: 1, pET21(+); 2, pCPP2211; 3, pCPP2210.

The apparent colinear arrangement of this group of *hrp* and *ysc* g nes led us to inspect the *P. s.* pv. *syringae* and *P. s.* pv. *tomato* HrpD proteins for possible similarity to the *Yersinia* spp. YscK proteins. The similarity between the HrpD of *P. s.* pv. *syringae* and *Y. pseudotuberculosis* was the highest, with 28% of the amino acids identical and 57% similar. The HrpD and YscK proteins are of similar overall composition, and they lack any predicted transmembrane segments. However, there is a striking discrepancy between the sizes of the two proteins. HrpD is only 133 amino-acids long, whereas YscK from *Y. pseudotuberculosis* is 209 amino-acids long. From the T7 experiments described above it is important to note that in the absence of a strong ribosome binding site, the precise initiation codon of the *hrpD* ORF is uncertain; it is conceivable that *hrpD* actually initiates immediately downstream of *hrpC*, at the ATG codon which overlaps the stop codon of *hrpC*, which would yield a predicted protein of 176 amino acids for $HrpZ_{Pss}$ or 175 amino acids for $HrpZ_{Pst}$ in an arrangement similar to that of the *yscJ* and *yscK* ORFs in *Yersinia* spp. However, this codon and all other potential initiation codons upstream of the one we have chosen lack ribosome binding sites, and the pattern of codon usage suggests that the intergenic region is not translated.

Although the similarities between HrpB/YscI, HrpD/YscK, and HrpE/YscL are lower than those involving HrpC/YscJ, the similarities of HrpB/YscI and HrpE/YscL are clearly in-

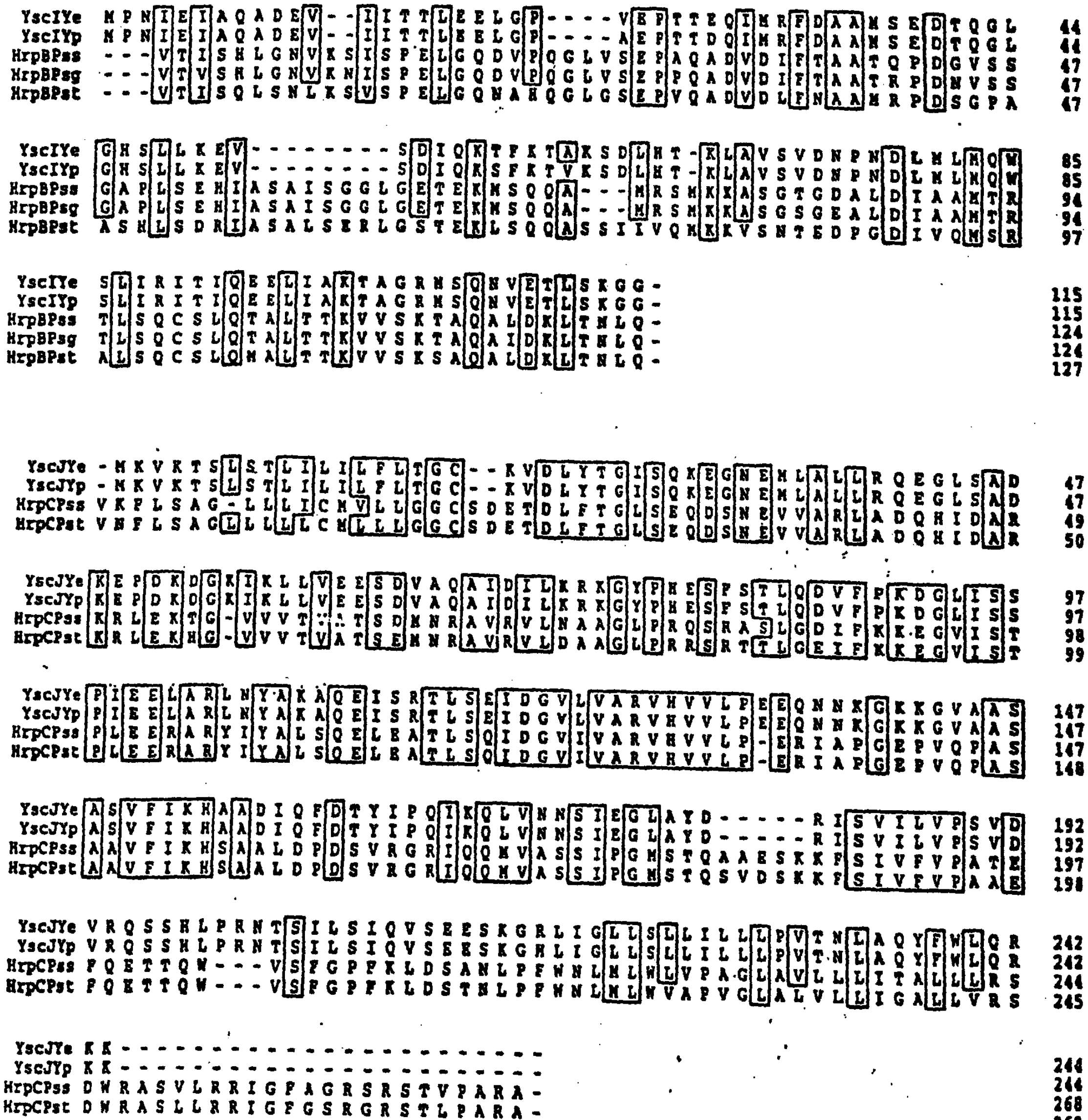


Fig. 6. Alignment of the protein sequences of HrpB from *Pseudomonas syringae* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato*, and HrpC, HrpD and HrpE from *P. s.* pv. *syringae* and *P. s.* pv. *tomato* with YscI, YscJ, YscK, and YscL from *Y. enterocolitica* and *Y. pseudotuberculosis* (Michiels et al. 1991; Rimpilainen et al. 1992). (continued on next page)

dicative of probable homology as based on a difference between the scores for the optimized and the average of 100 random Gap alignments being at least 5 times the standard deviation for the randomized alignments (Doolittle 1986). The scores for HrpD/YscK lie at the margin of significance by this measure. However, the varying levels of similarity are consistent with the divergence observed between Hrp proteins from different *P. syringae* pathovars and between Ysc proteins from different *Yersinia* spp. The results for HrpB,C and E lend support to the weak homology of HrpD to YscK and suggest that *hrpB*, *hrpC*, *hrpD*, and *hrpE* are colinear with *yscI*, *yscJ*, *yscK*, and *yscL*.

In a recent report, Van Gijsegem et al. (1995) observe that the *P. solanacearum* GMI1000 *hrp* cluster also encodes homologs of YscJ and YscL but not YscI and YscK. It is possible that with relatively divergent Hrp sequences, similarities with Ysc proteins may be found only after examining the sequences from several plant pathogens. It is interesting to note that there is no ORF following *hrpE* that is homologous to the protein encoded by the final gene of the *virC* operon, YscM. However, the *hrpZ* operon lies immediately upstream of the *hrpH* operon (Fig. 1), and HrpH is a homolog of YscC, a secretion protein which lies upstream of *yscIJKL* within the *virC* operon (Michiels et al. 1991). This suggests that a sig-

```
YscKYe   MMENYITSFQLRFCPAAYLHLEQLPSLWRSILPYLPQWRDSAHLNAALLD   50
YscKYp   MMENYITSFQLRFCPAAYLHLEQLPSLWRSILPYLPQWRDSA--NAALLD   48
HrpDPss  ----------------------------------------MVSRHSVFLQ   10
HrpDPst  ----------------------------------------MASRHGVFLQ   10

YscKYe   EFSLDTDYEEPHGLGALPLQPQSQLELLLCRLGLVLHGEAIRRCVLASPL  100
YscKYp   EFSLDTDYEEPHGLGALPLQPQSPLELLLCRLGLVLHGEAIRRCVLASPL   98
HrpDPss  SIGIT------------PSQPPHPAEPVLNWLALT-----------PVQR   37
HrpDPst  SLGID------------PAQPPAPAEPVLRWLALT-----------PSQR   37

YscKYe   QQLLTLVNQETLRQIIVQHELLIGPWPTNWQRPLPTEIESRTMIQSGLAF  150
YscKYp   --LLTLVNQETLRQIIVQHELLIGPWPTHWQRPLPTEIESRTMIQSGLAF  146
HrpDPss  DQALDLAQRICFSR--NESDGHDGQWCWALTKALRPGV--WLELEREDAR   83
HrpDPst  EQALSLAQCICFSR--NESDGPDGQWCWGLTKALRPGV--WLEFEHEDAR   83

YscKYe   WLAAKEPQPQAWCKRLSLRLPLATPSEPWLVAESQRPLAQTLCHKLVKQV  200
YscKYp   WLAAKE--PQAWCKRLSLRLPLATPSEPWLVAESQRPLAQTLCHKLVKQV  194
HrpDPss  LLLGAWLGPEYWSR---LRLAWAPDEVTDRPCAAPENKLQTLWQAVLWRV  130
HrpDPst  LLLGAWLGPQYWSR---LCLECPPNEVPDTPGKAPENKLQALWQAIMWRV  130

YscKYe   MPTCSHLFK                                           209
YscKYp   TPTCSHLFK                                           203
HrpDPss  TAT------                                           133
HrpDPst  TAA------                                           133
```

```
YscLYe   MSQTCQTGYAYMQPFVQIIPSNLSLACGLR--ILRAEDYQSSLTTEELIS   48
YscLYp   MSQTCQTGYAYMQPFVQIIPSNLSLACGLR--ILRAEDYQSSLTTEELIS   48
HrpEPss  --------------MLAKRSITLTADAVLPEPVLRREDIANSLLARDILT   36
HrpEPst  --------------MLAKRSIALTAATLLREPILRREDIADSLLARDILA   36

YscLYe   AAKQDAEKILADAQEVYEQQKQLGWQAGMDEARTLQATLIHETQLQCQQF   98
YscLYp   AAKQDAEKILADAQEVYEQQKQLGWQAGMDEARTLQATLIHETQLQCQQF   98
HrpEPss  DARRQAEQLLVLEQAKADHRHQ----EALAQFWERANAFLDELHVQREAL   82
HrpEPst  DARQQPTQILALEQEKAEHLQQ----QALAQFWENANAFLGELQVQREAL   82

YscLYe   YRHVEQQNSEVVLLAVRKILNDYDQVDMTLQVVREALALVSNQKQVVVRV  148
YscLYp   YRHVEQQNSEVVLLAVRKILNDYDQVAMTLQVVREALALVSNQKQVVVRV  148
HrpEPss  QQQAMTAVEELLTEALCQLLDETTLAERARALVRNLAASQLNEAVATLSV  132
HrpEPst  QEQAMTAVEELLSESLRHLLDDTTLAERARALARNLPSNQLNEAVATLSV  132

YscLYe   NPDQAGTIREQIAKVHKDFPEISYLEVTADARLDQGGCILETEVGIIDAS  198
YscLYp   NPDQAGAIREQIAKVHKDFPEISYLEVTADARLDQGGCILETEVGIIDAS  198
HrpEPss  HPEMAEPVAEWLAESR--FAE--HWELKRDATLTTESLRLSDANGAFEID  178
HrpEPst  HPQIADPVAEWLADSR--FSE--HWQLKRDATIASDSLRLSDANGAFDIA  178

YscLYe   IDGQIEALSRAISTTLGQMKVTEEE                           223
YscLYp   IDGQIEALSRAISTTLGQMKVTE--                           221
HrpEPss  WATLRNGLAGAEPAA----------                           193
HrpEPst  WADLRKGLLGVEPAA----------                           193
```

Fig. 6. *(continued from preceding page)*

nificant proportion of the *virC* operon is conserved in *P. syringae*, albeit in a rearranged form. Eckhardt (1978) gels of total DNA, Southern-blotted and probed with a 0.75-kb *BstXI* internal fragment of *hrpZ*$_{Pss}$, suggested that the *hrp* genes are chromosomal in the three strains of *P. syringae* studied, rather than being plasmid-borne as are the *hrp* genes of *P. solanacearum* GMI1000 or the *ysc* genes of *Yersinia* spp. (Van Gijsegem et al. 1993; data not shown). The homologies of the *hrpZ* operons are summarized in Table 1.

Overexpression, purification, and biological assay of HrpZ$_{Pst}$ and HrpZ$_{Psg}$.

Partially purified lysates of *E. coli* expressing HrpZ$_{Pst}$ and HrpZ$_{Psg}$ elicited a clear HR on tobacco while control lysates of *E. coli* containing vector alone did not. However the activity of the cell lysates on the two host plants was more ambiguous. Soybean is generally unreactive to cell lysates from either pathogen, while tomato is quite sensitive and sometimes weakly reactive not only to cell lysates of *E. coli* expressing HrpZ, but also to control lysates of *E. coli* containing vector alone. To accurately evaluate the biological properties of HrpZ from each of the two pathovars, it was necessary to purify HrpZ. It was also necessary to ascertain that the HR observed on tobacco was due solely to HrpZ and not to the products of either of the two flanking ORFs, HrpA and HrpB, since HrpA and a fusion protein of HrpB were being expressed in addition to HrpZ by the original *hrpZ*$_{Psg}$ and *hrpZ*$_{Pst}$ clones.

As a first step towards purifying HrpZ, we attempted to increase the level of expression. From the sequence of the *PstI* clones encoding *hrpZ* it was clear that long stretches of DNA encoding *hrpA* and the 3′ end of *hrpS* (1,144 bp in *hrpZ*$_{Psg}$+ pCPP2202 and 809 bp in *hrpZ*$_{Pst}$+ pCPP2203) separated *hrpZ* from the *lac* promoter in pBluescript II. A series of deletions of the 5′ end of the *hrpZ*$_{Pst}$ clone were constructed using the Erase-a-Base system (Promega), bringing the *lac* promoter within 100 bp of the *hrpZ* initiation codon, and removing *hrpA*. Although cell lysates expressing the deleted clones retained HR eliciting activity, they did not show a substantial increase in gene expression. Searching for an explanation for this behavior we identified a number of potential contributing factors. The first possibility was the presence of a *cis*-acting sequence contained in the 100 bp remaining upstream of *hrpZ*$_{Pst}$. Using a terminator analysis program we identified a 9-bp inverted repeat located between *hrpA* and *hrpZ* (Fig. 3). Although this repeat lacks the AT-rich sequence downstream which is characteristic of many terminators, it is possible that its presence encourages premature transcription termination. Similar repeats, albeit with weaker secondary structure, can be found upstream of *hrpZ*$_{Pss}$ and *hrpZ*$_{Psg}$. A second factor contributing specifically to the low expression of *hrpZ*$_{Pst}$ may be the absence of a strong ribosome binding site. Finally, there could be factors related to the proteins themselves, such as a lack of stability.

To eliminate possible *cis*-acting sequences and to obtain clones of *hrpZ*$_{Pst}$ and *hrpZ*$_{Psg}$ that lack *hrpA* and *hrpB*, the *hrpZ* genes from both pathovars were amplified by PCR, directionally cloned into pBluescript II and transformed into *E. coli* DH5α F′*lacI*^{q}. We obtained significantly increased expression of HrpZ$_{Psg}$ using the plasmid pCPP2255 (Fig. 7), but unexpectedly, overexpression of HrpZ$_{Pst}$ appeared to be deleterious to the cells, and plasmids recovered from transformants often showed rearrangements. To maximize expression of HrpZ$_{Pst}$ under these conditions, we introduced subclones containing the gene behind the T7 promoter of pET21(+) (Novagen, Madison, WI). Unlike the *lac* promoter, the T7 promoter is less sensitive to distance effects, and expression of HrpZ$_{Pst}$ in *E. coli* BL21(DE3), with pET21(+) as the vector, resulted in increased expression as shown in Figures 2 and 8. Expression in BL21(DE3) also allowed us to retain almost complete repression of *hrpZ* until induction with IPTG. Good expression of HrpZ$_{Pst}$ was achieved using the plasmid pCPP2211 in *E. coli* BL21(DE3).

The quality of the samples obtained following partial purification of the lysates by heat treatment was quite variable. To ensure removal of the majority of the contaminating proteins and to obtain a more concentrated sample of protein, we further purified HrpZ by ammonium sulphate precipitation and hydrophobic chromatography, which as indicated in Figure 8, yielded a distinct band on a Coomassie-stained gel. Purified, active HrpZ could then be obtained by electroelution from excised gel slices. This procedure was also used to isolate

Table 1. Homologies of *Pseudomonas syringae* pv. *syringae hrpZ* operon proteins with proteins from other *P. syringae* pathovars and *Yersinia* spp.

P. s. pv. *syringae*	HrpA (108)[a]	HrpZ (341)	HrpB (124)	HrpC (268)	HrpD (133)[d]	HrpE (193)
P. s. pv. *glycinea*	(108) 91/92[b]	(345) 79/87	(124) 94/96			
P. s. pv. *tomato*	(108) 28/42	(370) 63/75	(124) 68/80	(268) 90/95	(133) 78/87	(193) 76/87
Y. enterocolitica			YscI (115) 22/45[c] 24/45	YscJ (244) 35/59 38/60	YscK (203) 26/53 22/48	YscL (223) 21/47 22/46
Y. pseudotuberculosis			(115) 22/45 21/44	(244) 35/59 38/60	(209) 28/57 23/49	(221) 21/47 22/46

[a] Number of amino acids in the protein is given in parentheses.
[b] Percent identical and similar amino acids in comparison with the *P. s.* pv. *syringae* protein.
[c] The first pair of values are the percent identical and similar amino acids in comparison with the *P. s.* pv. *syringae* protein; the second are in comparison with *P. s.* pv. *tomato*.
[d] The data presented here are for the shorter of the two potential ORFs encoding *hrpD*. The larger versions of the HrpD proteins of *P. s.* pv. *syringae* and *P. s.* pv. *tomato* would be respectively 175 and 176 amino acids long with 74/84% identity/similarity to each other.

HrpZ from the supernatants of *P. s.* pv. *tomato* and *P. s.* pv *glycinea* grown in *hrp*-inducing minimal media (Fig. 9). Preparations of the purified HrpZ proteins from *P. s.* pvs. *syringae*, *glycinea*, and *tomato*, at a concentration of ≥20 µM in MES buffer, were infiltrated into the leaves of tobacco, soybean, and tomato. The three proteins elicited a collapse involving >50% of the infiltrated tissue in tobacco and tomato leaves that developed within 18 h and was typical of the HR elicited by incompatible *P. syringae* strains, but they caused no visible reaction in soybean. It is worth noting that tobacco and tomato plants vary substantially in their sensitivity to harpin preparations. For example, some leaves on sensitive tomato plants will respond to 2 to 5 µM $HrpZ_{Pst}$, but ≥20 µM is required for consistent results. Furthermore, unlike tobacco, tomato plants that have responded hypersensitively to a HrpZ preparation do not respond to subsequent infiltrations of the elicitor. The spurious necroses sometimes observed were deduced to result from mechanical damage incurred during infiltration or the infiltration of preparations contaminated with salts r containing high concentrations of vector control *E. coli* lysates. These necroses developed much more quickly (within 4 to 6 h), and were much weaker and patchier than the confluent HR elicited by HrpZ. The fact that the HR induced by HrpZ in tomato and tobacco is an active response of host tissu was confirmed by coinfiltration of either sodium vanadate at $5^{-5} \times 10^{-3}$ M or lanthanum chloride at 1 × 10 M. Each of these two inhibitors of plant metabolism completely inhibited the HR elicited by HrpZ preparations from each of the three pathovars but not the necrosis caused by the other factors mentioned.

DISCUSSION

We have used the *P. s.* pv. *syringae* 61 *hrpZ* gene to isolate the *hrpZ* locus from *P. s.* pv. *glycinea* race 4 and *P. s.* pv. *tomato* DC3000. Characterization of the *hrpZ* genes, products, and flanking DNA of these three pathovars has revealed the structure of the *hrpZ* operon, the relative variation among ORFs within the peron, the presence of genes downstream of *hrpZ* that are colinear with a block of genes involved with *Yersinia* virulence protein secretion, and the presence in $HrpZ_{Pss}$ of a sequence related to a sequence in the PopA1

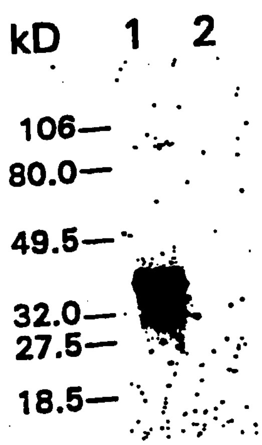


Fig. 7. Overexpression of $HrpZ_{Psg}$ in *E. coli* DH5α F′*lacI*^{Q}. Cultures were grown overnight at 30°C in LM with 1 mM IPTG. Cell lysates were partially purified by heat treatment, separated on an SDS-polyacrylamide gel, transferred to Immobilon-P, immunoblotted with anti-$HrpZ_{Pss}$ antibodies, and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase. Lanes: 1, *E. coli* DH5α F′*lacI*^{Q} (pCPP2255); 2 *E. coli* DH5α F′*lacI*^{Q} (pBluescript II).

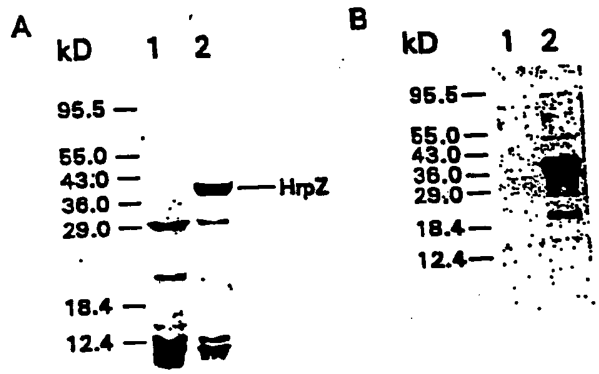


Fig. 8. Overexpression and purification of $HrpZ_{Pst}$. Cultures were grown to an OD_{600} of 0.6 and induced with 1 mM IPTG. $HrpZ_{Pst}$ was then partially purified from the cell lysate in a three-step process: first, by heat-treatment at 100°C as previously described, then by precipitation with ammonium sulphate at 30 to 45% saturation, and finally by binding to a hydrophobic resin (phenyl-sepharose) at 30% ammonium sulphate. A, Coomassie stained SDS-polyacrylamide gel. Lanes: 1, *E. coli* BL21(DE3)(pET21+); 2, *E. coli* BL21(DE3)(pCPP2211). B, Immunoblot of the samples shown in A, probed with anti-$HrpZ_{Pss}$ antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase.

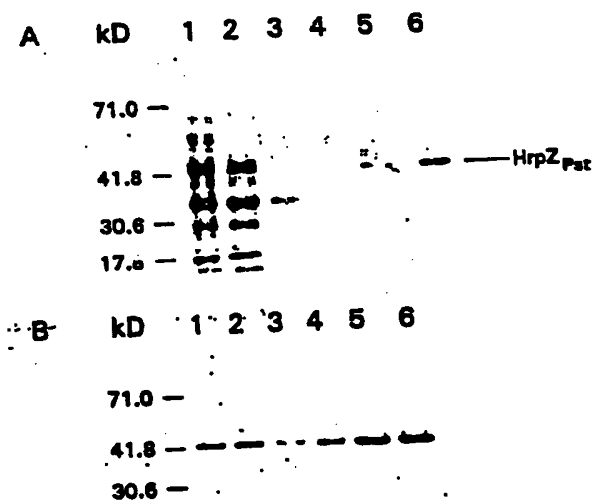


Fig. 9. Purification of $HrpZ_{Pst}$ from *hrp*-induced *Pseudomonas syringae* pv. *tomato*. Cells were grown in King's broth (KB) at 30°C and then resuspended in *hrp*-inducing minimal medium (Huynh et al. 1989) and incubated at room temperature overnight. Cells were removed by centrifugation and the supernatant heat-treated at 100°C for 10 min. Proteins in the supernatant were precipitated with ammonium sulphate at the percent saturations indicated. Proteins were desalted, concentrated, and resuspended in 5 mM MES using Centricon-10 tubes (Amicon). A, Coomassie stained SDS-polyacrylamide gel. Lanes: 1, supernatant extracted with Strataclean resin (Stratagene); 2, heat-treated supernatant extracted with Strataclean resin (Stratagene); 3, 0 to 20% ammonium sulphate fraction; 4, 20 to 30% ammonium sulphate; 5, 30 to 40% ammonium sulphate; 6, 30 to 45% ammonium sulphate. B, Immunoblot of the samples shown in A, probed with anti-$HrpZ_{Pss}$ antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase.

protein of the tomato pathogen *P. solanacearum* GMI1000. We also observed that purified $HrpZ_{Pn}$ was at least as effective as $HrpZ_{Pss}$ and $HrpZ_{Psg}$ in eliciting an HR-like necrosis in the leaves of tomato, a host of *P. s.* pv. *tomato* DC3000, whereas none of the HrpZ preparations elicited significant necrosis in soybean, the host of *P. s.* pv. *glycinea*.

The HrpZ proteins of three *P. syringae* pathovars.

A comparison of the sequences of the three HrpZ proteins with each other and with HR elicitors characterized from other bacteria indicates that the HrpZ proteins represent a distinct family of elicitors that is conserved among *P. syringae* pathovars. The amino acid sequences of the three proteins are sufficiently similar to reveal their relatedness, but (with the exception of a sequence within $HrpZ_{Pst}$), they show no significant relatedness to elicitor proteins from other bacteria. Interestingly, *hrpZ* is the second most divergent ORF in the *hrpZ* operons of *P. s.* pv. *syringae* and *P. s.* pv. *tomato*, with only 63% of the predicted amino acids being identical. Nevertheless, $HrpZ_{Pss}$, $HrpZ_{Psg}$, and $HrpZ_{Pst}$ are indistinguishable in several biological and physical properties. They have the same effect on different plants (discussed below), and they are heat stable, glycine-rich, and devoid of cysteine and tyrosine. The lack of tyrosine is a feature they differentially share with the *P. solanacearum* PopA1 protein but not the *Erwinia* harpins. This property has been speculated to allow the protein to avoid the H_2O_2-mediated cross-linking of tyrosine residues that may occur in plant cell walls during defense responses (Bradley et al. 1992; He et al. 1993).

Interestingly, a 24 amino acid, glycine-rich stretch of $HrpZ_{Pst}$ shows homology to part of PopA1, as does the cognate nucleotide sequence. The region of homology between $HrpZ_{Pst}$ and PopA1 corresponds exactly to the insertion in $HrpZ_{Pst}$. The insertion of this element within $HrpZ_{Pst}$ sequences that are otherwise similar among the three HrpZ proteins suggests horizontal transfer and a common ancestry with PopA1. Because the host range of *P. solanacearum* overlaps with that of *P. s.* pv. *tomato*, it is tempting to speculate that this region has some particular significance to pathogenesis on tomato, although, as discussed below, this is not obvious from the different effects of the two proteins on tomato.

The presence of this insert in active $HrpZ_{Pst}$ is another indicator of the apparent plasticity of structure/function relationships in these glycine-rich elicitor proteins. That significant changes to the structure of these proteins does not abolish their activity was previously demonstrated when a fortuitous $hrpZ_{Pss}$ clone was found to produce an active derivative of HrpZ missing the N-terminal 125 amino acids, and the *popA* product was observed to be degraded in culture to an active form missing the N-terminal 93 amino acids (He et al. 1993; Arlat et al. 1994). Clearly the presence of this "additional" internal sequence does not diminish the ability of the protein to elicit the HR. In fact, although it is difficult to make a quantitative assessment, $HrpZ_{Pst}$ may actually be a slightly more potent elicitor of the HR than $HrpZ_{Pss}$.

However, $HrpZ_{Pst}$ appears to differ from the other HrpZ proteins in being deleterious to *E. coli* cells when overexpressed and is possibly more unstable, making it difficult to purify large amounts of the protein. Since the glycine-rich region is the most obvious difference between $HrpZ_{Pst}$ and $HrpZ_{Pss}$ it is possible that it contributes to this phenomenon. We were able to overcome this problem experimentally by using a tightly regulated T7 promoter/polymerase system, but never obtained quite the same level of expression we achieved with $HrpZ_{Pss}$ and $HrpZ_{Psg}$. However, there remains the obvious question of how HrpZ toxicity is avoided by *P. s.* pv. *tomato*. One possibility would be that HrpZ is never expressed at levels high enough to affect the bacterium, even when it is induced in planta. Some indirect evidence for this hypothesis is provided by our examination of the DNA upstream of $hrpZ_{Pst}$. The ORF has a weak ribosome binding site, and we also observed that expression of cloned *hrpZ* from the *lac* promoter appears to be attenuated by the presence of *cis*-acting upstream sequences. A 9-bp GC-rich repeat upstream of *hrpZ* may be significant in this regard. Preliminary data from northern blotting experiments also indicate that premature transcription termination may take place when *hrpA-hrpZ* clones are expressed in *E. coli* (G. Preston, unpublished). A second possibility is that the location of the *hrpZ* gene in an operon with secretion genes ensures tight coupling of synthesis and secretion. Genes encoding extracellular proteins and secretion pathway components are often coregulated, but with a few exceptions involving the type I pathway, they do not lie within the same operon (Fath and Kolter 1993). A third possibility is that *P. s.* pv. *tomato* is more tolerant of high levels of HrpZ than is *E. coli*, or it possesses a means of keeping HrpZ in a nontoxic form while it is in the cell.

Further comparison with the *Yersinia* virulence system presents an intriguing possibility in this regard. It has been shown that secretion of certain "Yops" (the *Yersinia* pathogenicity determinants), involves chaperone proteins, small hydrophilic proteins which help keep the Yop protein in a translocation competent form and help target it for secretion (Wattiau et al. 1994). The genes encoding each chaperone are located adjacent to the gene encoding the corresponding Yop. Given the presence of several small ORFs of undetermined function in the pHIR11 *hrp* cluster, it is tempting to speculate that one of them, particularly *hrpA*, might encode a protein with chaperone function. There is a superficial resemblance between HrpA and *Yersinia* chaperones such as SycE. They are all small, hydrophilic, cytoplasmic proteins which lack a signal sequence, but there are no specific homologies. We are now constructing nonpolar mutations to test the role of HrpA in secretion. Preliminary results suggest that HrpA is not required for *E. coli* MC4100(pHIR11) to elicit an HR or secrete HrpZ (J. R. Alfano, unpublished), but in chaperone-mediated systems limited secretion of a protein will usually occur even in the absence of its chaperone, so it may be necessary to look quantitatively at secretion and accumulation of HrpZ to assess whether mutations in *hrpA* or other *hrp* genes have an effect.

The colinear relationship between several *hrp* and *ysc* genes.

From the sequence of the *hrpZ* operon it is clear that the parallels with the *Yersinia* type III secretion pathway extend beyond homologies of individual genes. The four genes downstream of *hrpZ*, *hrpB–E*, appear to be arranged colinearly with the region of the *virC* secretion operon from *Yersinia* that encodes YscI–L. The *virC* operon is a large operon containing 13 genes, *yscA-yscM*, several of which have been demonstrated to have a role in Yop secretion (Michiels et al. 1991). Of the four *Yersinia* genes with putative ho-

mologs in the *hrpZ* operon, only *yscJ* and *yscL* are known to have a role in secretion. An accompanying paper shows that five more *hrp* genes, downstream of the *hrpH* operon, are colinear with the *yscQ–U* genes in the *virB* operon of *Yersinia* (Huang et al. 1995).

It appears that a significant proportion of the type III secretion pathway described in *Yersinia* can be identified in *P. syringae*, and it seems likely that increasing parallels between the two systems will be found. In both systems the secreted proteins are involved with early events in the interaction with the host, and expression of secretion genes and virulence proteins is tightly coregulated. The secretion pathway seems to function in a similar way, as in both cases secreted proteins lack an N-terminal signal peptide and are not posttranslationally processed.

HrpZ and host specificity.

The function of HrpZ in compatible interactions is unclear. A likely role is the release of nutrients to the apoplast. Atkinson and Baker (1987a, 1987b) have proposed that the alkalinization of the apoplast caused by Hrp+ bacteria (which occurs at a slower rate in compatible interactions) results in the leakage of sucrose and other nutrients to support bacterial growth. One of the key unanswered questions regarding the *P. syringae* HrpZ proteins is their role in host specificity. Compatible interactions leading to disease are distinguished by the absence of the HR. Host-differential elicitor activity would be one way to reconcile the production of HR-eliciting proteins by *P. syringae* and the phenomenon of host-specific compatibility. The failure of the PopA1 protein to elicit the HR in tomato, a host of *P. solanacearum* GMI1000, supports this concept (Arlat et al. 1994). Similarly, the isolated *P. s.* pv. *syringae* 61 HrpZ protein fails to elicit the HR in bean, although the significance of this is diminished by the fact that bean leaves appear insensitive to any harpins (He et al. 1993). To further explore this question, we infiltrated all three HrpZ proteins into the leaves of the host plants for each of the pathovars. The host plants of *P. s.* pv. *syringae* 61, and *P. s.* pv. *glycinea*, bean and soybean, respectively, are uniformly unreactive to HrpZ from both compatible and incompatible pathogens; however, tomato leaves proved to be highly sensitive to all three HrpZ proteins. Thus, our data argue against the hypothesis that host-differential activity of HrpZ proteins controls the host specificity of *P. syringae* pathovars.

If isolated $HrpZ_{Pst}$ elicits the HR in tomato, why does *P. s.* pv. *tomato* not elicit the HR during pathogenesis? One possibility is that the response of tomato to $HrpZ_{Pst}$ is qualitatively different than the response to $HrpZ_{Pss}$ and $HrpZ_{Psg}$ despite manifestation of the same gross morphology. That is, the necrosis elicited by $HrpZ_{Pst}$ is fundamentally different than the HR and does not involve associated defenses that stop the pathogen. We are now testing this possibility with probes for HR-specific transcripts. A second possibility is that $HrpZ_{Pst}$ production is regulated in a host-specific manner. However, *hrpZ* is clearly part of the Hrp regulon: *hrpZ* expression is transcriptionally linked with genes encoding components of the secretion pathway, the *hrpZ* operons in all three of these *P. syringae* pathovars have virtually the same *hrp/avr* promoter sequence, and expression of the *hrpZ* operon is likely required for pathogenicity. Th conserved promoter sequences suggests that the *hrpZ* operon is regulated in *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* by the same nutritional conditions and HrpR, HrpS, HrpL regulatory cascade described for *P. s.* pv. *syringae* and *P. s.* pv. *phaseolicola* (Grimm and Panopoulos 1989; Rahme et al. 1992; Xiao et al. 1992; Xiao et al. 1994; Xiao and Hutcheson 1994; Grimm et al. 1995). Whether differential expression of the Hrp regulon controls host specificity awaits determination. A third possibility is that the *P. syringae* pathovars produce host-specific suppressors of defense responses. This is supported by the observation that compatible pathogens do not trigger defense responses in host plants that are elicited by nonpathogens (Jakobek et al. 1993).

It is important to note that our data do not eliminate the possibility that the three HrpZ proteins actually have differential activity in host plants when delivered by living bacteria and that the HR observed may be an abnormal response resulting from the presentation of a high concentration of HrpZ in an artificial manner. In that regard, it is interesting that legumes, which appear insensitive to isolated harpins, respond to Hrp recombinant *E. coli* cells that secrete the same proteins (He et al. 1993). Experiments in which the *hrpZ* genes of *P. syringae* pathovars are switched or altered in their patterns of deployment should test more definitively the role of HrpZ in determining host specificity.

In conclusion, we have characterized an operon containing two components of the Hrp+ system of *P. syringae*—a block of secretion-related genes that are conserved in eukaryotic pathogens in the genera *Pseudomonas*, *Xanthononas*, *Erwinia*, *Yersina*, *Shigella*, and *Salmonella* and a gene encoding an elicitor that is unique to plant pathogens. The elicitors found in the *P. syringae* pathovars are a subfamily of a larger class that appears to be characteristic of plant pathogens, and which we postulate to have a role in releasing nutrients for bacterial utilization. Our challenge now is to determine how the various components of the Hrp system have been adapted to serve plant parasitism in the face of plant defenses.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Bacteria and plasmids used in this study are shown in Table 2.

Pseudomonads were routinely grown in King's B broth (King et al. 1954) at 30°C, but for certain experiments the *hrp*-derepressing minimal medium of Huynh et al. (1989), adjusted to pH 5.5, was used. *E. coli* was grown in LM (Sambrook et al. 1989) or terrific broth (Tartof and Hobbs 1987). Plasmids were introduced into bacteria by transformation (Sambrook et al. 1989) or electroporation (Gene Pulser, Bio-Rad).

Plant materials.

The plants used in this study were tobacco (*Nicotiana tabacum* L. 'Xanthii'), tomato (*Lycopersicon esculentum* Mill. 'Moneymaker'), and soybean (*Glycine max* L. 'Harosoy'). Plants were grown in a greenhouse or growth chamber at 23° to 25°C with a photoperiod of 16 to 24 h. Infiltration of plant leaves with HrpZ preparations was performed with blunt syringes as described (Huang et al. 1988).

DNA analysis and sequencing.

All DNA manipulations, except where specified, followed standard protocols (Ausubel et al. 1987; Sambrook et al. 1989). The *hrpZ* region of pHIR11 was subcloned into

pBluescript II (Huang et al. 1995). Two *Pst*I fragments of 2.2 and 2.4 kb from pCPP2201 and pCPP2200, respectively, were subcloned into pBluescript II SK(–) in both orientations. A series of overlapping nested deletions covering both strands was generated for each of the subclones using Erase-a-Base (Promega, Madison, WI). The deletions were sequenced from double-stranded templates using Sequenase version 2.0 (U.S. Biochemicals, Cleveland, OH) and forward and reverse M13 primers. Sequencing was completed using specific primers synthesized by Integrated DNA Technologies (Coralville, IA). In addition, the 3.7 and 3.6 kb *Sac*I-*Eco*RI fragments, which overlap the *Pst*I subclones from pCPP2201 and pCPP2200, were also subcloned into pBluescript II SK(–) and sequenced using the ABI 373A DNA sequencer at the Cornell Biotechnology Program DNA sequencing facility and specific primers synthesized by IDT. Nucleotide and derived amino acid sequences were analyzed with the Genetics Computer Group Sequence Analysis Software Package (Devereaux et al. 1984). Homology searches against major sequence databases were done with the BLAST program (Altschul et al. 1990).

PCR amplification of *hrpZ* from *P. s.* pv. *glycinea* and *P. s.* pv. *tomato*.

The *hrpZ* genes of *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* were amplified by PCR from the plasmids pCPP2202 and pCPP2203, respectively. Reactions were performed using the PCR Optimizer kit (Invitrogen, San Dieg , CA) according to the manufacturer's instructions. Reactions were overlaid with mineral oil and incubated in a Hybaid Thermal Reactor (Hybaid, Teddington, U.K.) using these cycle parameters: 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 2 min at 55°C, 3 min at 72°C, followed by a final incubation of 7 min at 72°C. The primers used for $hrpZ_{Psg}$ were 5′-TACGGGATCCTTTGAGGAGGTTGTGATG-3′ and 5′-TACGCTGCAGTATC AGTCAGGCAGCAGC-3′, and those for $hrpZ_{Pst}$ were 5′-TACGGGATCCATGCAAGCACTTA ACAGC-3′ and 5′-GGAACTGCAGCAAGCTCCGGCGA-TACAC-3′. All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and were designed to introduce a *Bam*HI and a *Pst*I site at the 5′ and 3′ ends, respectively, of each amplified fragment.

The $hrpZ_{Psg}$ fragment from pCPP2202 was successfully amplified in all reaction buffers tested. The $hrpZ_{Pst}$ fragment from pCPP2203 was successfully amplified using reaction buffer B (reaction concentration 60 mM Tris-HCl, 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, pH 8.5). PCR products of the expected sizes of 1.0 and 1.2 kb were purified from an agarose gel, digested with *Pst*I and *Bam*HI, cloned into pBluescript II, and then transformed into *E. coli* DH5α F′*lac*I, yielding plasmid pCPP2255 carrying $hrpZ_{Psg}$. Plasmids containing

Table 2. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Reference or source
Escherichia coli		
DH5α	*supE*44 Δ*lac*U169 (φ80*lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 Nal^r	Hanahan 1983; Life Technologies, Inc. Grand Island, NY
DH5α F′*lac*I[Q]	F′ *pro*AB[+] *lac*I[q]ZΔM15 zzf::Tn5[Km^r]/φ80d *lac*ZΔM15 Δ(*lac*ZYA-*argF*)U169 *end*A1 *rec*A1 *hsd*R17 ($r_k^- m_k^+$) *deo*R *thi*-1 *sup*E44λ⁻ *gyr*A96 *rel*A1	Life Technologies Inc.
BL21(DE3)	F⁻ *omp*T *hsd*B_B ($r_B^- m_B^-$) *dcm gal* DE3	Novagen
Pseudomonas syringae		
pv. *syringae* 61	Wild type	Baker *et al.* 1987
pv. *glycinea* race 4	Wild type	C. J. Baker
pv. *tomato* DC3000	Wild type, Rp^r	D. E. Cuppels
Plasmids		
pBluescript II SK(-)	Cloning vector, Amp^r	Stratagene
pUCP19	pUC19 derivative, Amp^r	Schweizer 1991
pET21(+)	T7 transcription vector, Amp^r	Novagen
pT7-6	T7 transcription vector; Amp^r	Tabor and Richardson 1988
LITMUS 28	Cloning vector, Amp^r	New England Biolabs
pHIR11	25-kb cosmid containing *P.s.* pv. *syringae* 61 *hrp* cluster	Huang *et al.* 1988
pSYH10	$hrpZ_{Pss}$ ORF in pBluescript II	He *et al.* 1993
pCPP2303	0.8-kb *Pst*I-*Age*I subclone from pHIR11, containing *hrpB*, in LITMUS 28	This study
pCPP2305	1.3-kb *Sal*I-*Sac*I subclone from pHIR11, containing *hrpD*, in pT7-6	This study
pCPP2200	pUCP19 carrying 10-kb partial *Sau*3A1 fragment of *P. s.* pv. *glycinea* DNA with $hrpZ_{Psg}$	This study
pCPP2202	2.4-kb *Pst*I subclone of pCPP2200 in pBluescript II: $hrpA_{Psg}$ and $hrpZ_{Psg}$ in expressed orientation with respect to P_{lac}	This study
pCPP2204	As pCPP2202 but with $hrpZ_{Psg}$ in reversed orientation to P_{lac}	This study
pCPP2206	2.4-kb *Pst*I $hrpA_{Psg}$ and $hrpZ_{Psg}$ subclone from pCPP2202 in pET21(+)	This study
pCPP2208	3.6-kb *Sac*I-*Eco*RI $hrpZ_{Psg}$ subclone from pCPP2200 in pBluescript II	This study
pCPP2210	1.85-kb *Bgl*II-*Pst*I $hrpZ_{Psg}$ subclone from pCPP2202 in pET21(+)	This study
pCPP2255	PCR-amplified $hrpZ_{Psg}$ ORF in pBluescript II	This study
pCPP2201	pUCP19 carrying 10-kb fragment of *P. s.* pv. *tomato* DNA with $hrpZ_{Pst}$	This study
pCPP2203	2.2-kb *Pst*I subclone of pCPP2201 in pBluescript II; $hrpA_{Pst}$ and $hrpZ_{Pst}$ in expressed orientation with respect to P_{lac}	This study
pCPP2205	As pCPP2203 but with $hrpZ_{Pst}$ in reversed orientation to P_{lac}	This study
pCPP2207	2.2-kb $hrpZ_{Pst}$ subclone from pCPP2203 in pET21(+)	This study
pCPP2209	3.7-kb *Sac*I-*Eco*RI subclone from pCPP2201 containing $hrpBCDE_{Pst}$ in pBluescript II	This study
pCPP2304	3.7-kb *Sac*I-*Eco*RI subclone from pCPP2209 in LITMUS 28	This study
pCPP2211	2.0-kb *Bgl*II-*Pst*I $hrpZ_{Pst}$ subclone from pCPP2203 in pET21(+)	This study

[a] Amp^r = ampicillin resistance; Nal^r = nalidixic acid resistance; Rp^r = rifampicin resistance.

PCR-amplified $hrpZ_{Pss}$ w re found to be unstable and appeared to promote cell lysis.

HrpZ purification and analysis.

HrpZ was purified from *E. coli* as previously described (He et al. 1993) with the following modifications. Cells were lysed in ither 5 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.5, or cell lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0). For some experiments the supernatant from heat-treated lysate was partially purified after sonication by ammonium sulphate precipitation (25 to 45% saturation), with desalting and concentration being performed with Centricon-10 tubes (Amicon). For experiments requiring highly purified HrpZ expressed in *E. coli* BL21(DE3), the supernatant was further purified by binding to phenyl-sepharose (Sigma) in the presence of ammonium sulphate (>30% saturation) and elution with 5 mM MES, pH 5.5, followed by electrophoresis through a native 15% polyacrylamide gel. The purified protein was then eluted from excised gel slices using an Elutrap apparatus (Schleicher & Schuell) or from crushed gel slices using a Micropure separator (Amicon). Protein concentrations were determined using Bio-Rad protein assay solution. HrpZ was also purified from heat-treated supernatants of *P. syringae* grown in *hrp*-inducing medium (Huynh et al. 1989) by ammonium sulphate precipitation (25 to 45% saturation) and desalting/concentration using Centricon-10 tubes. For infiltration into plant tissue, HrpZ preparations were diluted to various degrees with 5mM MES, pH 5.5. The amino-terminal sequence analyses were performed at the Cornell Biotechnology Program Protein Analysis Facility ($HrpZ_{Psg}$) and the University of Kentucky Macromolecule Structure Analysis Facility ($HrpZ_{Pss}$).

T7 expression and labeling of proteins in *E. coli*.

Proteins encoded by the *hrpZ* operon were expressed in *E. coli* BL21(DE3) by using the pET21(+) T7 expression system (Novagen). Conditions for isopropyl-β-D-thiogalactopyranoside (IPTG) induction of T7 RNA polymerase-dependent expression and labeling with L-[^{35}S]methionine were as described by Studier et al. (1990). After being labeled, cells were collected by centrifugation and then resuspended and lysed in SDS-loading buffer and the proteins resolved on an SDS-polyacrylamide gel. Gels were stained, dried and exposed to Kodak X-ray film.

Nucleotide sequence accession numbers.

The nucleotide sequences reported in this paper have been deposited in GenBank under accession numbers L41861 (*P. syringae* pv. *tomato hrpA, hrpZ, hrpB, hrpC, hrpD, hrpE*), L41862 (*P. syringae* pv. *glycinea hrpA, hrpZ, hrpB*), L41863 (*P. syringae* pv. *syringae hrpA*), and L41864 (*P. syringae* pv. *syringae hrpB*).

ACKNOWLEDGMENTS

We thank Kent Loeffler for the photography and Frédérique Van Gijsegem and Christian Boucher for sending us their manuscript before publication. This work was supported by NSF grant MCB 9305178.

LITERATURE CITED

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Arlat, M., Van Gijsegem, F., Huet, J. C., Pernollet, J. C., and Boucher, C. A. 1994. PopA1, a protein which induces a hypersensitive-like response on specific *Petunia* genotypes, is secreted via the Hrp pathway of *Pseudomonas solanacearum*. EMBO J. 13:543-553.

Atkinson, M. M., and Baker, C. J. 1987. Association of host plasma membrane K⁺/H⁺ exchange with multiplication of *Pseudomonas syringae* pv. *syringae* in *Phaseolus vulgaris*. Phytopathology 77:1273-1279.

Atkinson, M. M., and Baker, C. J. 1987. Alteration of plasmalemma sucrose transport in *Phaseolus vulgaris* by *Pseudomonas syringae* pv. *syringae* and its association with K⁺/H⁺ exchange. Phytopathology 77:1573-1578.

Ausubel, F. M., Brent, R., Kingston, R., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1987. Current Protocols in Molecular Biology. John Wiley & Sons, New York.

Baker, C. J., Atkinson, M. M., and Collmer, A. 1987. Concurrent loss in Tn*5* mutants of *Pseudomonas syringae* pv. *syringae* of the ability to induce the hypersensitive response and host plasma membrane K⁺/H⁺ exchange in tobacco. Phytopathology 77:1268-1272.

Bauer, D. W., Wei, Z.-M., Beer, S. V., and Collmer, A. 1995. The *Erwinia chrysanthemi* EC16 $harpin_{Ech}$: A protein that is required for elicitation of the hypersensitive response and full virulence. Mol. Plant-Microbe Interact. 8:484-491.

Beer, S. V., Bauer, D. W., Jiang, X. H., Laby, R. J., Sneath, B. J., Wei, Z.-M., Wilcox, D. A., and Zumoff, C. H. 1991. The *hrp* gene cluster of *Erwinia amylovora*. Pages 53-60 in: Advances in Molecular Genetics of Plant-Microbe Interactions. H. Hennecke and D. P. S. Verma, ed. Kluwer Academic Publishers, Dordrecht.

Bonas, U. 1994. *hrp* genes of phytopathogenic bacteria. Pages 79-98 in: Current Topics in Microbiology and Immunology, Vol. 192: Bacterial Pathogenesis of Plants and Animals—Molecular and Cellular Mechanisms. J. L. Dangl, ed. Springer-Verlag, Berlin.

Bonas, U., Schulte, R., Fenselau, S., Minsavage, G. V., Staskawicz, B. J., and Stall, R. E. 1991. Isolation of a gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and the hypersensitive response on pepper and tomato. Mol. Plant-Microbe Interact. 4:81-88.

Boucher, C. A., Van Gijsegem, F., Barberis, P. A., Arlat, M., and Zischek, C. 1987. *Pseudomonas solanacearum* genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. J. Bacteriol. 169:5626-5632.

Bradley, D. J., Kjelvom, P., and Lamb, C. J. 1992. Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. Cell 70:21-30.

Collmer, A., Bauer, D. W., Alfano, J. R., Preston, G., Loniello, A. O., Huang, H.-C., and He, S. Y. 1994. The role of *Pseudomonas syringae* and *Erwinia chrysanthemi hrp* gene products in plant interactions. Pages 49-56 in: Advances in Molecular Genetics of Plant-Microbe Interactions, Vol. 3. M. J. Daniels, ed. Kluwer, Dordrecht.

Devereaux, J., Haeberli, P., and Smithies, O. 1984. A comprehensive set of sequence analysis programs for the VAX. Gene 12:387-395.

Doolittle, R. F. 1986. Of Urfs and Orfs: A primer on how to analyze derived amino acid sequences. Mill Valley, CA.

Eckhardt, T. 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid 1:584-588.

Fath, M. J., and Kolter, R. 1993. ABC transporters: Bacterial exporters. Microbiol. Rev. 57:995-1017.

Fenselau, S., Balbo, I., and Bonas, U. 1992. Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. Mol. Plant-Microbe Interact. 5:390-396.

Gough, C. L., Genin, S., Zischek, C., and Boucher, C. A. 1992. *hrp* genes of *Pseudomonas solanacearum* are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. Mol. Plant-Microbe Interact. 5:384-389.

Grimm, C., and Panopoulos, N. J. 1989. The predicted protein product of a pathogenicity locus from *Pseudomonas syringae* pv. *phaseolicola* is homologous to a highly conserved domain of several procaryotic regulatory proteins. J. Bacteriol. 171:5031-5038.

Grimm, G., Aufsatz, W., and Panopoulos, N. J. 1995. The *hrpRS* locus of *Pseudomonas syringae* pv. *phaseolicola* constitutes a complex regulatory unit. Mol. Microbiol. 15:155-165.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166:557-580.

He, S. Y., Huang, H.-C., and Collmer, A. 1993. Pseudomonas syringae pv. syringae harpin$_{Pss}$: A protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. Cell 73:1255-1266.

Huang, H.-C., He, S. Y., Bauer, D. W., and Collmer, A. 1992. The *Pseudomonas syringae* pv. *syringae* 61 *hrpH* product: An envelope protein required for elicitation of the hypersensitive response in plants. J. Bacteriol. 174:6878-6885.

Huang, H.-C., Hutcheson, S. W., and Collmer, A. 1991. Characterization of the *hrp* cluster from *Pseudomonas syringae* pv. *syringae* 61 and Tn*phoA* tagging of genes encoding exported or membrane-spanning Hrp proteins. Mol. Plant-Microbe Interact. 4:469-476.

Huang, H.-C., Lin, R.-W., Chang, C.-J., Collmer, A., and Deng, W.-L. 1995. The complete *hrp* gene cluster of *Pseudomonas syringae* pv. *syringae* 61 includes two blocks of genes required for harpin$_{Pss}$ secretion that are arranged colinearly with *Yersinia ysc* homologs. Mol. Plant-Microbe Interact. 8:733-746.

Huang, H. C., Schuurink, R., Denny, T. P., Atkinson, M. M., Baker, C. J., Yucel, I., Hutcheson, S. W., and Collmer, A. 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco. J. Bacteriol. 170:4748-4756.

Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: Regulation of a pathogen gene determining host cultivar specificity. Science 245:1374-1377.

Innes, R. W., Bent, A. F., Kunkel, B. N., Bisgrove, S. R., and Staskawicz, B. J. 1993. Molecular analysis of avirulence gene *avrRpt2* and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes. J. Bacteriol. 175:4859-4869.

Jakobek, J. L., Smith, J. A., and Lindgren, P. B. 1993. Suppression of bean defense responses by *Pseudomonas syringae*. Plant Cell 5:57-63.

Jones, C. J., Homma, M., and Macnab, R. M. 1989. L-, P-, and M-ring proteins of the flagellar basal body of *Salmonella typhimurium*: Gene sequences and deduced protein sequences. J. Bacteriol. 171:3890-3900.

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Med. 22:301-307.

Klement, Z. 1963. Rapid detection of pathogenicity of phytopathogenic pseudomonads. Nature 199:299-300.

Klement, Z., Farkas, G. L., and Lovrekovich, L. 1964. Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathology 54:474-477.

Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. 1986. Gene cluster of *Pseudomonas syringae* pv. *"phaseolicola"* controls pathogenicity of bean plants and hypersensitivity on nonhost plants. J. Bacteriol. 168:512-522.

Lonetto, M., Gribskov, M., and Gross, C. A. 1992. The σ^{70} family: Sequence conservation and evolutionary relationships. J. Bacteriol. 174:3843-3849.

Meinhardt, L. W., Krishnan, H. B., Balatti, P. A., and Pueppke, S. G. 1993. Molecular cloning and characterization of a *sym* plasmid locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA257. Mol. Microbiol. 9:17-29.

Michiels, T., Vanooteghem, J.-C., de Rouvroit, C. L., China, B., Gustin, A., Boudry, P., and Cornelis, G. R. 1991. Analysis of *virC*, an operon involved in the secretion of Yop proteins by *Yersinia enterocolitica*. J. Bacteriol. 173:4994-5009.

Pugsley, A. P. 1989. Protein Targeting. Academic Press, New York.

Rahme, L. G., Mindrinos, M. N., and Panopoulos, N. J. 1992. Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syringae* pv. phaseolicola. J. Bacteriol. 174:3499-3507.

Rimpilainen, M., Forsberg, A., and Wolf-Watz, H. 1992. A novel protein, LcrQ, involved in the low-calcium response of *Yersinia pseudotuberculosis* shows extensive homology to YopH. J. Bacteriol. 174:3355-3363.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning. A Laboratory Manual, Second Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schweizer, H. P. 1991. *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. Gene 97:109-112.

Shen, H., and Keen, N. T. 1993. Characterization of the promoter of avirulence gene D from *Pseudomonas syringae* pv. tomato. J. Bacteriol. 175:5916-5924.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 185:60-89.

Tabor, S., and Richardson, C. C. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA 82:1074-1078.

Tartof, K. D., and Hobbs, C. A. 1987. Improved media for growing plasmid and cosmid clones. Bethesda Res. Lab. Focus 9:12.

Van Gijsegem, F., Genin, S., and Boucher, C. 1993. Evolutionary conservation of pathogenicity determinants among plant and animal pathogenic bacteria. Trends Microbiol. 1:175-180.

Van Gijsegem, F., Gough, C., Zischek, C., Niqueux, E., Arlat, M., Genin, S., Barberis, P., German, S., Castello, P., and Boucher, C. 1995. The *hrp* gene locus of *Pseudomonas solanacearum*, which controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex. Mol. Microbiol. 15:1095-1114.

Wattiau, P., Bernier, B., Deslee, P., Michiels, T., and Cornelis, G. R. 1994. Individual chaperones required for Yop secretion by *Yersinia*. Proc. Natl. Acad. Sci. USA 91:10493-10497.

Wei, Z.-M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A., and Beer, S. V. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science 257:85-88.

Xiao, Y., Hue, S., Yi, J., Lu, Y., and Hutcheson, S. W. 1994. Identification of a putative alternate sigam factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. syringae Pss61 *hrp* and *hrmA* genes. J. Bacteriol. 176:1025-1036.

Xiao, Y., and Hutcheson, S. 1994. A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae*. J. Bacteriol. 176:3089-3091. Author's correction. 176:6158.

Xiao, Y., Lu, Y., Heu, S., and Hutcheson, S. W. 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 *hrp* cluster. J. Bacteriol. 174:1734-1741.

Turner JG, Novacky A (1974) The quantitative relation between plant and bacterial cells involved in the hypersensitive reaction. Phytopathology 64: 885–90
Van Gijsegem F (1986) Analysis of the pectin-degrading enzymes secreted by three strains of Erwinia chrysanthemi. J Gen Microbiol 132: 617–624
Van Gijsegem F (1989) Relationship between the pel genes of the pelADE cluster in Erwinia chrysanthemi strain B374. Mol Microbiol 3: 1415–1424
Van Gijsegem F, Genin S, Boucher C (1993) Evolutionary conservation of pathogenicity determinants among plant and animal pathogenic bacteria. Trends Microbiol 1: 175–180
Vayda ME, Schaeffer HJ (1988) Hypoxic stress inhibits the appearance of wound-response proteins in potato tubers. Plant Physiol 88: 805–809
Vayda ME, Antonov LS, Yang Z, Butler WO, Lacy GH (1992) Hypoxic stress inhibits aerobic wound-induced resistance and activates hypoxic resistance to bacterial soft rot. Am Potato J 69: 239–253
Wandersman C (1989) Secretion, processing and activation of bacterial extracellular proteases. Mol Microbiol 3: 1825–1831
Wandersman C, Delepelaire P, Letoffe S, Schwartz M (1987) Characterization of Erwinia chrysanthemi extracellular proteases: cloning and expression of the protease genes in Escherichia coli. J Bacteriol 169: 5046–5053
Wei Z-M, Laby RJ, Zumoff CH, Bauer DW, He SY, Collmer A, Beer SV (1992) Harpin, elicitor of the hypersensitive response produced by the plant pathogen Erwinia amylovora. Science 257: 85–88
·llen MC, Inness RW, Bent AF, Staskawicz BJ (1991) Identification of Pseudomonas syringae pathogens of Arabidopsis and a bacterial locus determining avirulence on both Arabidopsis and soybean. Plant Cell 3: 49–59
Willis DK, Barta TM, Kinscherf (1991a) Genetics of toxin production and resistance in phytopathogenic bacteria. Experientia 47: 765–771
Willis DK, Rich JJ, Hrabak EM (1991b) hrp genes of phytopathogenic bacteria. Mol Plant Microbe Interact 4: 132–138
Xiao Y, Lu Y, Heu S, Hutcheson SW (1992) Organization and environmental regulation of the Pseudomonas syringae pv. syringae 61 hrp cluster. J Bacteriol 174: 1734–1741
Yamazaki N, Fry SC, Darvill AG, Albersheim P (1983) Host-patahogen interactions. XXIV. Fragments isolated from suspension-cultured sycamore cell walls inhibit the ability of the cells to incorporate [^{14}C] leucine into proteins. Plant Physiol 72: 864–869
Yang Z, Cramer CL, Lacy GH (1992) Erwinia carotovora subsp. carotovora pectic enzymes: In planta gene activation and roles in soft-rot pathogenesis. Mol Plant Microbe Interact 5: 104–112
Yoder MD, Keen NT, Jurnak F (1993) New domain motif: the structure of pectate lyase C, a secreted plant virulence factor. Science 260: 1503–1507
Young JM (1974) Development of bacterial populations in vivo in relation to plant pathogenecity. NZ J Agric Res 17: 105–113
Yucel I, Xiao Y, Hutcheson SW (1989) Influence of Pseudomonas syringae culture conditions on initiation of the hypersensitive response of cultured tobacco cells. Appl Environ Microbiol 55: 1724–1729
Zink RT, Engwall JK, McEvoy JL, Chatterjee AK (1985) recA is required in the induction of pectin lyase and carotovoricin in Erwinia carotovora subsp. carotovora. J Bacteriol 164: 390–396
·ker M, Hankin L (1970) Regulation of pectate lyase synthesis in Pseudomonas fluorescens and Erwinia carotovora. J Bacteriol 104: 13–18
Zucker M, Hankin L, Sands D (1972) Factors governing pectate lyase synthesis in soft rot and non-soft rot bacteria. Physiol Plant Pathol 2: 59–67

AC 2728

hrp G n s of Phytopathogenic Bacteria

U. Bonas

Bonas, U. 1994. *hrp* genes of phytopathogenic bacteria. Fages 79-98 in: Current Topics in Microbiology and Immunology, Vol. 192: Bacterial Pathogenesis of Plants and Animals - Molecular and Cellular Mechanisms. J. L. Dangl, ed. Springer-Verlag, Berlin.

1 Introduction

In nature plants are resistant to the majority of pathogens, and many bacteria live in close contact with the plant without causing any harm (see chapter by Beattie and Lindow in this volume). Among the 1600 different speci s known in the bacterial kingdom only a small number (about 80) are plant pathogenic and in most cases highly specialized with respect to the plant that can be attacked. Only a few of these species are gram-positive, e.g., *Clavibacter* ssp. and *Streptomyces* ssp. In this review I focus on subspecies of the gram-negative genera *Erwinia*, *Pseudomonas*, and *Xanthomonas*, which comprise the major bacterial plant pathogens.

To be a successful pathogen the invading bacterium has to overcome the plant's defense. During evolution plant pathogenic bacteria have acquired multiple functions that enable them to colonize and multiply in living plant tissue. In nature, bacteria enter the plant through natural openings (stomata, hydathodes) or

CNRS Institut des Sciences Végétales, Avenue de la Terrasse, 91198 Gif-sur-Yvette, France

wounds. The bacterial armory contains a number of weapons that contribute to pathogenicity. Obvious examples include degradative extracellular enzymes such as pectinases, cellulases, and proteases. When the corresponding genes are mutat d, bacterial ability to invade plant tissues is more or less affected depending on the pathogen, i.e., these functions contribute to and modulate development and severity of infection to different extents (see chapters by Dow and Daniels, and Collmer and Bauer in this volume).

In addition, phytopathogenic bacteria possess a large number of genes needed for basic pathogenicity. These genes have been operationally defined as *hrp* (hypersensitive reaction and pathogenicity; LINDGREN et al. 1986) based on their mutant phenotype. *hrp* genes are not only essential for pathogenicity on a plant, i.e., the ability to cause disease in a compatible interaction, but also for the incompatible interaction with resistant host varieties or with plants that are not normally a host for the particular pathogen (so called non-host). The incompatible nteraction is often associated with the induction of a hypersensitive reaction (HR) n the plant. In contrast to the use of the term hypersensitivity in the animal field, in plants the HR is a rapid defense response involving localized plant cell death, production of phenolics and antimicrobial agents, e.g., phytoalexins, at the site of infection (KLÉMENT 1982; LINDSAY et al. 1993). The HR results in prevention of pathogen multiplication and spread and thus in prevention of disease development. Under natural infection conditions the HR is microscopically small and can be induced by just one bacterial cell. Only when bacteria are introduced into plant tissue at high cell densities in the laboratory (about 10^7 colony forming units or m re/ml) is the HR macroscopically visible as confluent necrosis and can be clearly distinguished from typical disease symptoms. It is important to note that saprophytic or nonpathogenic bacteria such as *Escherichia coli* or *Pseudomonas fluorescens* do not induce the HR and are unable to multiply in plant tissue.

2 Isolation of *hrp* Genes and General Features

rp genes have been isolated from all major gram-negative plant pathogenic bacteria except Agrobacterium. There are excellent reviews that describe the early work or focus more on one particular pathogen (WILLIS et al. 1991; BOUCHER et al. 1992). The majority of *hrp* genes have been identified by complementation of loss-of-function mutants. Mutants obtained by random chemical (e.g., N-methyl-*N'*-nitro-*N*-nitrosoguanidine) or transposon mutagenesis of a pathogenic wild-type strain were inoculated into the host plant and screened for loss of both the ability to cause disease in susceptible plants and to induce the HR in resistant host or non-host plants (often tobacco). The second criterion for the isolation of genes specific for the plant interaction was to ensure that the mutants would still grow in minimal medium. This way mutants affected in genes for basic housekeeping functions were eliminated. A third characteristic of all *hrp* mutants is that they are unable to grow in the plant.

The *hrp* genes were originally described for the bean pathogen *Pseudomonas syringae* pv. *phaseolicola*. LINDGREN and coworkers (1986) isolated Tn*5*-induced mutants of *P.s.* pv. *phaseolicola* that had lost both the ability to induce halo-blight disease on bean and the HR in tobacco. Complementation with cosmid clones from a genomic library of the wild-type strain resulted in isolation of a cluster of *hrp* genes localized in a 20 kb DNA region. This was the first indication that both the ability to cause disease and to induce the HR are mediated by common steps in a "pathway".

Since then *hrp* gene clusters have been cloned from a number of different bacteria. Examples include *Pseudomonas solanacearum* (BOUCHER et al. 1987; Fig. 1B), the *Xanthomonas campestris* pathovars *campestris* and *vitians* (ARLAT et al. 1991), *translucens* (WANEY et al. 1991), and *vesicatoria* (BONAS et al. 1991; Fig. 1A), *Erwinia amylovora* (STEINBERGER and BEER 1988; BARNY et al. 1990; WALTERS et al. 1990; BAUER and BEER 1991), and several other pathovars of *P. syringae* (e.g., HUANG et al. 1988; LINDGREN et al. 1988; Fig. 1C). In addition, genes with DNA homology, and in some cases functional homology, have been isolated from other species, e.g., the so-called *wts* genes from *E. stewartii* (COPLIN et al. 1992; LABY and BEER 1992), and a region containing pathogenicity genes from *X.c.* pv. *glycines* that complement *hrp* mutants of *X.c.* pv. *vesicatoria* (HWANG et al. 1992; Bonas, unpublished results). Interestingly, nonpathogenic xanthomonads that were originally isolated from diseased plants as opportunists together with pathogenic bacteria do not contain *hrp*-related DNA sequences (STALL and MINSAVAGE 1990; BONAS et al. 1991). In *Agrobacterium tumefaciens* or in strains of *Rhizobium* ssp. there seem to be no *hrp* gene equivalents present (BONAS et al. 1991; LABY and BEER 1992). This conclusion is based on DNA hybridization experiments and, of course, does not exclude the presence of genes with functional homology to *hrp* genes in these species.

In all of the cases mentioned above, the *hrp* genes are organized in clusters of 22–40 kb, and I will restrict most of this chapter to these large *hrp* clusters. In addition, several smaller *hrp* loci have been described that are not linked to the large cluster present in the same bacterium. These include a region in *P. solanacearum* (HUANG et al. 1990), the *hrpX* locus that is conserved in *X. campestris* pathovars *campestris* (KAMOUN and KADO 1990; KAMOUN et al. 1992) and *oryzae* (KAMDAR et al. 1993), and the *hrpM* locus in *P.s.* pv. *syringae* (NIEPOLD et al. 1985; MUKHOPADHYAY et al. 1988). *hrpM* is functionally conserved in pathovar *phaseolicola* (FELLAY et al. 1991). Besides being nonpathogenic and unable to induce the HR in tobacco, *P. syringae hrpM* mutuants are also affected in mucus production. The *hrpM* locus encodes two putative proteins which are similar and have been shown to be functionally homologous to the products of the *E. coli mdoGH* operon (LOUBENS et al. 1993). The *mdoGH* genes are required for periplasmic membrane-derived oligosaccharide synthesis in *E. coli*. The *hrpQ* and *hrpT* genes from *P.s.* pv. *phaseolicola* (MILLER et al. 1993) will be discussed later in this chapter.

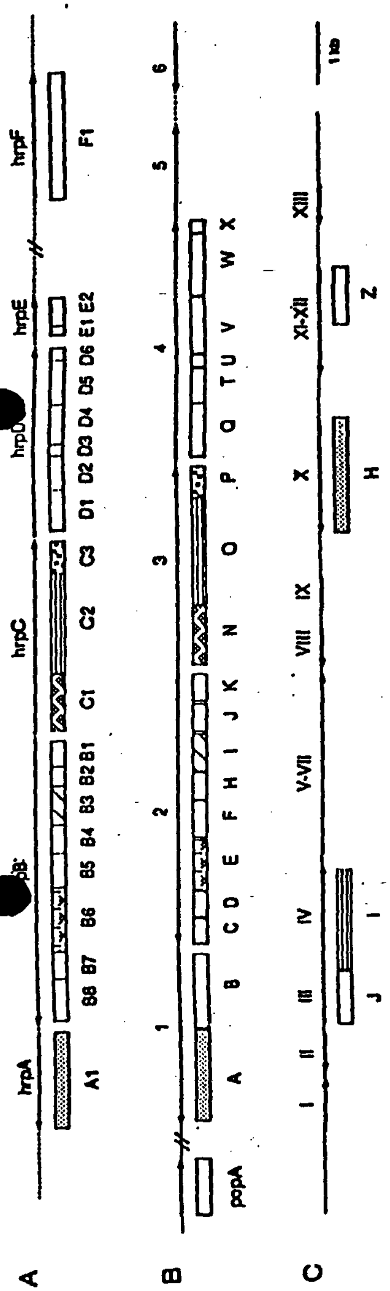


Fig. 1A–C. Genetic and translational organization of the *hrp* gene cluster of different plant pathogenic bacteria. A *Xanthomonas campestris* pv. *vesicatoria*; B *Pseudomonas solanacearum*; and C *Pseudomonas syringae* pv. *syringae*. Arrows represent transcription units as determined by genetic analyses. Boxes correspond to sequences of open reading frames (ORFs) that have been published. In case of sequence similarities between ORFs in different clusters the boxes are filled with the same pattern. For references, see text

3 Structural Organizati n and Relat dn ss of *hrp* Clusters

Genetic studies using transposon-induced insertion mutants in the respective bacterial wild-type strains revealed that the *hrp* clusters contain at least six to eight complementation groups (Fig. 1). Some *hrp* gene clusters have clearly been shown to be localized in the chromosome, e.g., in *P.s.* pv. *phaseolicola* (RAHME et al. 1991) and in *X.c.* pv. *vesicatoria* (BONAS et al. 1991), whereas in *P. solanacearum*, the *hrp* cluster is on a megaplasmid (BOUCHER et al. 1987).

Striking similarities have recently been found between the *hrp* genes of pathogens belonging to different genera. The first indication of homologies came from Southern hybridization studies. DNA homology was observed among different strains of the same pathovar, as well as between pathovars or strains within a species, and in some cases also between species. However, the degree of conservation varies. DNA homology is high within pathovars of a given subspecies, e.g., in *P. syringae* (LINDGREN et al. 1988; HUANG et al. 1991) and in *X. campestris* (BONAS et al. 1991). The latter studies were recently extended by PCR using primers based on *hrp* sequences from *X.c.* pv. *vesicatoria* (LEITE et al. 1994). Furthermore, at least some regions of the *hrp* clusters appear to be conserved on the DNA level between *P. solanacearum* and pathovars of *X. campestris*, *P. syringae*, and also to *E. amylovora* (BOUCHER et al. 1987; ARLAT et al. 1991; GOUGH et al. 1992; LABY and BEER 1992). In addition, cross-complementation within a subspecies indicated a high degree of functional conservation of *hrp* genes (e.g., LINDGREN et al. 1988; ARLAT et al. 1991; BONAS et al. 1991; LABY and BEER 1992). Due to sequence data it is now becoming more and more apparent that several *hrp* genes are conserved in all major gram-negative plant pathogenic bacteria (see below). Whether there are *hrp* genes that are clearly pathovar-specific can only be answered when complete sequence information becomes available for several *hrp* clusters.

4 Function of *hrp* Genes in *Xanthomonas campestris* pv. *vesicatoria* and Other Plant Pathogenic Bact ria

DNA sequence analysis of the *hrp* genes has revealed some important clues to their possible biochemical functions. One of the first genes sequenced was a regulatory gene, *hrpS*, from *P.s.* pv. *phaseolicola* (GRIMM and PANOPOULOS 1989). This gene as well as *hrpB*, a regulatory gene from *P. solanacearum* (GENIN et al. 1992), will be discussed below in the context of gene regulation.

Since *hrp* genes are environmentally regulated (see below), it was believed for a while that they would be encoding "alternative" proteins required for adaptation of the bacterium to the plant as the preferred environment. The recently discovered sequence similarities between several putative Hrp proteins and known proteins from other bacteria, however, led to a very different hypothesis, namely, involvement of Hrp proteins in protein secretion. We have

sequenced the entire *hrp* cluster of *X.c.* pv. *vesicatoria*. Since most *hrp* sequences from this and oth r bacteria are not yet published, I will summariz our results and refer to the other phytopathogenic bacteria as I go along. Based on genetic analyses and the open reading frames (ORFs) with a high coding probability we predict 21 *hrp* genes in the 25 kb *hrp* cluster of *X.c.* pv. *vesicatoria*. Their transcriptional organization is depicted in Fig.1A. The loci *hrpA* and *hrpB* are transcribed fr m right to left; the other four loci are transcribed from left to right (SCHULTE and BONAS 1992a). According to the locus (*hrpA–hrpF*) we have numbered the ORFs consecutively. The *hrpA* locus appears to contain just one *hrp* gene, *hrpA1*. The *hrpB* operon contains eight ORFs, called *hrpB1–hrpB8*, etc. A region f about 4 kb between *hrpE* and *hrpF* does not seem to be involved in the interaction with the plant because insertions in this region do not lead to a change in phenotype (BONAS et al. 1991).

What are the characteristics of the Hrp proteins? It should be noted that, except for three proteins, expression of the other 18 has yet to be demonstrated in *X.c.* pv. *vesicatoria*. A number of putative Hrp proteins are most likely associated with or localized in the bacterial membrane. For example, the HrpC2 protein sequence contains eight transmembrane domains but lacks a signal sequence, sugg sting an inner membrane localization (FENSELAU et al. 1992). Both HrpA1 and HrpB3 contain an NH_2-terminal signal sequence and one (HrpA1) or two (HrpB3) transmembrane domains, suggesting that a part of these proteins might be targeted to the outer membrane. The signal sequence of HrpB3 resembles signal peptidase II sequences which are typical of lipoproteins (FENSELAU t al. 1992). Experiments using radioactively labeled palmitate are underway to test whether HrpB3 is a lipoprotein. Recently, both HrpB3 and HrpA1 were shown to be localized in the *X.c.* pv. *vesicatoria* membrane fraction using polyclonal antibodies (S. Fenselau, C. Marie, and U. Bonas, manuscript in preparation). The HrpB6 protein is a putative ATPase with highly conserved nucleotide and magnesium binding domains. It is more similar to protein traffic ATPases than to proton pump ATPases, and the lack of membrane spanning domains suggests a cytoplasmic location (FENSELAU et al. 1992).

Searches of the database revealed sequence relatedness of more than half of the *X.c.* pv. *vesicatoria* Hrp proteins with putative proteins in other bacteria, including different plant pathogens. High DNA sequence identity (more than 90%) was found to a 2.7 kb fragment carrying pathogenicity genes from *X.c.* pv. *glycines* (HWANG et al. 1992). The authors predicted two ORFs, whereas in *X.c.* pv. *vesicatoria*, this region contains three ORFs corresponding to the *hrpC3*, *hrpD1* and *hrpD2* genes. Complementation studies indicated that part of the *hrp* region is colinear in the two pathovars of *Xanthomonas* (unpublished).

The deduced amino acid sequences of *hrp* genes published from *P. solanacearum* (GOUGH et al. 1992, 1993; GENIN et al. 1992) show significant similarity to *X.c.* pv. *vesicatoria* proteins (Table 1; Fig.1). One exception is the *hrpB* regulatory gene from *P. solanacearum* which is not present in the 25 kb *hrp* region or in the flanking region of the *X.c.* pv. *vesicatoria hrp* cluster as determined by DNA sequence analysis and hybridization studies (T. Horns and U. Bonas, unpublished). Furthermor , s veral of the proteins mentioned are conserved in other species (Fig.1), however, the degree of sequence similarity varies greatly (Table 1). The HrpA1 protein from *X.c.* pv. *vesicatoria* is 48% and 29% identical to proteins from *P. solanacearum* (HrpA; GOUGH et al. 1992) and *P.s.* pv. *syringae* (HrpH; HUANG et al. 1992), respectively. HrpC2 from *X.c.* pv. *vesicatoria* is even more conserved, being 66% idential to the corresponding HrpO protein of *P. solanacearum* (GOUGH et al. 1993), whereas the *hrpI* genes from *E. amylovora* (WEI and BEER 1994) and from *P.s.* pv. *syringae* (HUANG et al. 1993) both show 62% similarity to *hrpC2* from *X.c.* pv. *vesicatoria*. *P.s.* pv. *syringae* also contains a *hrpB3* related gene, called *hrpY*, and a *hrpD2* related gene, *hrpW* (H.-C. Huang, personal communication). Thus, the high degree of DNA sequence conservation that was reported earlier (see above) is also seen on the protein level. It appears that *hrp* genes in *X.c.* pv. *vesicatoria* are more closely related to *P. solanacearum* than to *P. syringae* and to *Erwinia*. As more and more homologous *hrp* genes are found in other bacteria nomenclature might become confusing. However, as long as the genes have not been shown to be functionally homologous, we will continue to use these names.

Besides genes that are conserved among the major phytopathogenic bacteria some genes are absent in the *hrp* region of more distantly related species. For example, there are no known homologs of the harpin genes *hrpN* (WEI et al. 1992a), and *hrpZ* (HE et al. 1993) (see below), and of *hrpJ* from *P.s.* pv. *syringae* (HUANG et al. 1993) in the *X.c.* pv. *vesicatoria hrp* cluster (unpublished; see Fig. 1).

Similarities of 50%–60% were found recently between HrpA1 and HrpB3 from *X.c.* pv. *vesicatoria* and two putative Nol proteins of *Rhizobium fredii* that are encoded by a cultivar specificity region. NolT and NolW mutants have a wider host range in nodulation of soybean (MEINHARDT et al. 1993). In addition, the authors mention that release of proteins is affected.

Last but not least, Table 1 summarizes the significant sequence similarities which have been found to proteins from animal bacterial pathogens. A number of putative Hrp proteins are related to proteins in animal pathogens such as *Salmonella*, *Shigella*, and *Yersinia* ssp. Since the first similarities found were to the Ysc, Vir, and Lcr proteins from *Yersinia* ssp, this group of organisms became a "role model" for plant pathologists (FENSELAU et al. 1992; GOUGH et al. 1992; HUANG et al. 1992). In *Yersinia*, these proteins are essential for the secretion of virulence factors, called Yops (Yersinia outer protein; MICHIELS et al. 1990, 1991). Since they are described in detail in the chapter by G.R. Cornelis, I will mention only a few important features. The Yops are hydrophilic proteins that lack a typical NH_2-terminal signal peptide, and are secreted by using an ntirely different pathway from that previously described for protein secretion. The genes involved in secretion are clustered on a 70 kb virulence plasmid. In case of a mutation, e.g., in *Yscj*, the Yops accumulate in the cytoplasm (MICHIELS et al. 1991). Although their direct role in transport has yet to be demonstrated, it is believed that the Ysc and Lcr proteins mentioned in Table 1 are parts of a special transport apparatus for Yop secretion. Similarly, *Shigella flexneri* secretes virulence factors, called Ipa (invasion plasmid antigens), that are distinct from Yops but share the general

Table 1. Sequence similarities of *Xanthomonas campestris* pv. *vesicatoria* Hrp proteins

Xanthomonas campestris pv. *vesicatoria*	HrpA1[1]	HrpB6[1]	HrpB3[1]	HrpC1[2]	HrpC2[1]	HrpC3[2]	HrpD1[2]	HrpD2[2]
Pseudomonas solanacearum	HrpA[3] (66%)	HrpE[4]	HrpI[3] (70%)	HrpN[5] (74%)	HrpO[3] (81%)	HpaP[5] (54%)	HrpQ[4]	HrpT[4]
Pseudomonas syringae pv. *syringae*	HrpH[6] (52%)				HrpI[7] (62%)			
Yersinia enterocolitica	YscC[8] (55%)		YscJ[8] (56%)					
Yersinia pestis	YscC[9] (55%)				LcrD[10] (70%)		LsaA[11] (52%)	LsaB[11] (72%)
Yersinia pseudotuberculosis		YscN[12] (73%)	LcrKa[13] (56%)					
Shigella flexneri	MxiD[14] (50%)	Spa47[15] (65%)	MxiJ[16] (52%)	Spa40[17] (55%)	MxiA[18] (65%)			Spa24[15] (67%)
Salmonella typhimurium	InvG[19] (52%)	SpaL[20] (70%) FliI[21] (65%)		SpaS[20] (56%)	InvA[22] (67%)			SpaP[20] (64%)
Bacillus subtilis		FlaA-ORF4[23] (68%)		FlhB[24] (62%)	FlhA[25] (63%)			FliP[26] (68%)
Escherichia coli		β-F1[27] (53%)						FliP[28] (65%)
Erwinia carotovora							MopB[29] (49%)	MopC[29] (65%)
Erwinia amylovora					HrpI[30] (62%)			
Rhizobium fredii	NolW[31] (51%)		NolT[31] (61%)					
Caulobacter crescentus					FlbF[32] (55%)			

Similarities between deduced amino acid sequences of Hrp proteins from *X.c.pv.vesicatoria* and other proteins include conservative amino acid exchanges. Number in parentheses indicates percent similarity.
Superscript numbers indicate references as follows:
1, Fenselau et al. 1992; 2, Bonas et al., unpublished; 3, Gough et al. 1992; 4, Genin et al. 1993, sequences unpublished; 5, Gough et al. 1993; 6, Huang et al. 1992; 7, Huang et al. 1993; 8, Michiels et al. 1991; 9, Haddix and Straley 1992; 10, Plano et al. 1991; 11, Fields et al. unpublished, accession # L22495; 12, Galyov, unpublished, accession # U00998; 13, Rimpilainen et al. 1992; 14, Allaoui et al. 1993; 15, Venkatesan et al. 1992; 16, Allaoui et al. 1992; 17, Sasakawa et al. 1993; 18, Andrews and Maurelli 1992; 19, Lodge et al., unpublished, accession # X75302; 20, Groisman and Ochman 1993; 21, Vogler et al. 1991; 22, Galán et al. 1992; 23, Albertini et al. 1991; 24, Carpenter et al., unpublished, accession # X741212; 25, Carpenter and Ordal 1993; 26, Bischoff et al. 1992; 27, Saraste et al. 1981; 28, Malakooti et al. unpublished, accession # L21994; 29, Mulholland et al. 1993; 30, Wei and Beer 1993; 31, Meinhardt et al. 1993; 32, Ramakrishanan et al. 1991; Sanders et al. 1992.

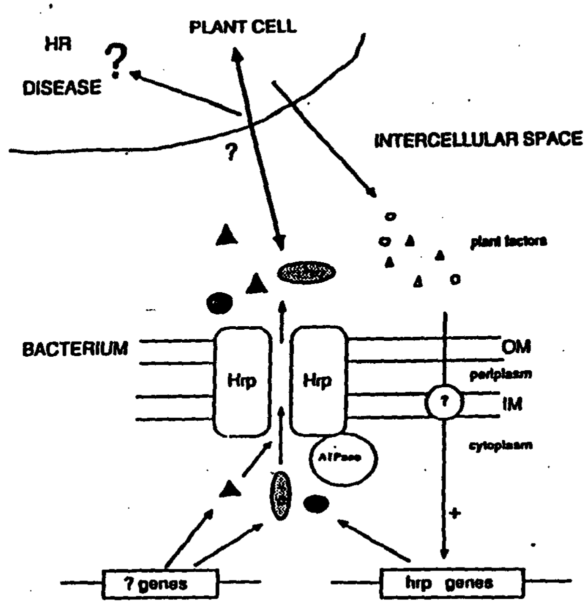


Fig. 2. Hypothetical model of cell signaling between gram-negative bacteria and plants indicating the proposed function of Hrp proteins as an apparatus for protein secretion. The model has been modified after FENSELAU et al. (1992). Hrp proteins may form a tunnel that enables the export of molecules such as virulence factors or avirluence factors leading to either a hypersensitive response (HR) or disease. These factors could be encoded by *hrp* genes or genes unlinked to the large cluster. Both types of genes have been found to encode elicitors of the HR (see text). The secretion of virulence proteins is hypothetical

features mentioned above (HALE 1991; and see chapter by PARSOT, this volume). Although *S. typhimurium* appears to possess a secretion system similar to that in *Shigella*, secreted invasion antigens have not yet been identified (GROISMAN and OCHMAN 1993; see chapter by FINLAY). As unpublished reports indicate that more and more genes in the animal pathogens are conserved, the data shown in Table 1 will soon be out of date. Proteins from other bacteria, e.g., *E. coli*, *Bacillus*, *Caulobacter* and from the *mop* region in *E. carotovora* (MULHOLLAND et al. 1993), have also been found to be similar to Hrp proteins (Table 1). Most of these are important for the assembly of the flagella, motility, or chemotaxis, again pointing, in my opinion, to a specialized secretion system rather than an involvement of *hrp* genes in chemotaxis.

These observations led us and others to propose a *hrp*-dependent secretion system in plant pathogenic bacteria (FENSELAU et al. 1992; GOUGH et al. 1992; VAN GIJSEGEM et al. 1993). A model is shown in Fig. 2 and raises certain questions, e.g., if secretion occurs, what is being secreted by plant pathogenic bacteria? So far, a few proteins have been identified as elicitors of the HR but there is no evidence for secretion of virulence factors (see below).

5 *hrp*-dependent Secretion of Hypersensitive Response-Inducing Proteins

5.1 Harpin from *Erwinia amylovora*

An important feature of the isolated *hrp* clusters from both *E. amylovora* and *P.s.* pv. *syringae* is the ability of *E. coli* or *Pseudomonas fluorescens* transformants containing the cloned genes to induce the HR on tobacco (HUANG et al. 1988; BEER et al. 1991; see below). This has prompted to search for the HR-inducing activity within the respective gene clusters.

The first bacterial HR-inducing protein identified, designated harpin, is a cell envelope-associated protein encoded by the *hrpN* gene of *E. amylovora*, a pathogen of pear and apple (WEI et al. 1992a). This harpin$_{Ea}$ is a glycine-rich and heat-stable protein that induces the HR in the non-host, tobacco. The *hrpN* gene is localized within the respective *hrp* cluster and thus has a dual role in also being required for pathogenicity on the normal host plant. Its function in pathogenicity, however, is unknown. BEER et al. (1993) mentioned in a preliminary report that the *hrpN* gene seems to be conserved among *Erwinia* ssp. but that there is no DNA homology between *hrpN* and sequences in the other plant pathogenic bacteria. Although data described below suggest that the harpin$_{Ea}$ protein might be secreted via the Hrp secretory apparatus, there is no published information available that demonstrates this.

5.2 Harpin from *Pseudomonas syringae* pv. *syringae*

Using an elegant approach He and coworkers recently have identified harpin$_{Pss}$, which is encoded by the *hrpZ* gene in the bean pathogen *P.s.* pv. *syringae* (HE et al. 1993; see Fig. 1C and chapter by Collmer and Bauer). Lysates of *E. coli* clones containing an expression library, made using the cloned *P.s.* pv. *syringae hrp* cluster, were directly screened for HR-inducing activity on tobacco leaves. Two proteins were identified, one of which was an NH_2-terminal deletion of harpin$_{Pss}$ with even higher activity than the full size protein. Whether or not processing occurs in natural infection is not clear. Interestingly, two short direct repeats in the COOH-terminus of harpin$_{Pss}$ are essential for elicitor activity. Although the two harpins harpin$_{Ea}$ and harpin$_{Pss}$ differ in their primary sequence, they have several features in common, e.g., a stretch of 22 amino acid that is similar in both proteins (HE et al. 1993). Harpin$_{Pss}$ is also glycine-rich and heat-stable. As with harpin$_{Ea}$ of *E. amylovora*, the function of harpin$_{Pss}$ in pathogenicity is unknown. Its product is secreted by *P.s.* pv. *syringae* in a HrpH-dependent way; HrpH is highly related to proteins involved in secretion in other plant and animal pathogens (HUANG et al. 1992; see Table 1).

5.3 *PopA* from *Pseudomonas solanacearum*

An HR-inducing protein has been identified and characterized from *P. solanacearum* culture supernatants, called Pop (Pseudomonas out protein; ARLAT et al. 1994). PopA1 and two shorter derivatives, PopA2 and PopA3, induce the HR in tobacco and in certain, but not all, *Petunia* lines. Like the harpins, the Pop proteins are also heat-stable and glycine-rich, however, the sequence is entirely different. In contrast to the harpins, the *popA* gene is not a *hrp* gene but is located outside of the large *hrp* cluster. Interestingly, expression of *popA* is *hrpB*-dependent, i.e., the gene is part of the *hrp* regulon. Mutations in *popA* do not affect the HR on tobacco or pathogenicity on tomato suggesting that more than on HR-inducing factor is produced. ARLAT et al. (1994) convincingly showed that secretion of PopA is dependent on other *hrp* genes, such as *hrpA*, *hrpN*, and *hrpO* (Fig. 1B). If a bacterial strain virulent towards *Petunia* is found it will be interesting to see if PopA acts as an avirulence protein in *Petunia* as has been suggested by the authors.

These xciting findings prove that certain Hrp proteins of *P.s.* pv. *syringae* and *P. solanacearum* play a role in transport of HR elicitors (Fig. 2). They also stimulate mor qu stions. It needs to be shown that harpins and PopA are in fact secreted when the bacteria interact with the plant (the *hrp* genes were induced in vitro). Are harpins conserved among pathovars of *P. syringae*? How many elicitors of the non-host HR in tobacco can be found? Is the mechanism of recognition in tobacco identical with the *Erwinia* and *P.s.* pv. *syringae* harpins and the *P. solanacearum* Pops?

6 R gulati n of Expression of *hrp* Genes

Expression of *hrp* genes is controlled by environmental conditions and has been studied on the RNA level as well as using transcriptional fusions to reporter genes such as the *E. coli* genes encoding β-galactosidase or β-glucuronidase. In general, expression of *hrp* loci is not detectable when the bacteria are grown in complex culture media. However, after growth of the bacteria in the plant, *hrp* genes are expressed. Attempts to mimic the conditions that the different bacterial species encounter in the plant tissue resulted in the finding that growth in minimal media without any plant-derived factor was sufficient to induce *hrp* genes. This has led to the speculation that the bacteria have to experience some kind of starvation conditions for full expression of *hrp* genes. One of the first indications for *hrp* gene expression in vitro, and clearly a breakthrough, was a report on the *hrp*-dependent expression of an avirulence gene from the soybean pathogen *P.s.* pv. *glycinea* (HUYNH et al. 1989).

Since the composition of minimal media differs depending on the bacterium studied, the most important findings will be summarized for representative pathogens. Parameters like carbon source, concentration of organic nitrogen and phosphate, osmolarity, and pH have been found to be important. High concentration of organic nitrogen generally appears to suppress *hrp* gene activation. Only two regulatory genes have been studied so far (see below). Interestingly, they both belong to different families of regulatory proteins.

6.1 *Pseudomonas syringae*

Expression of all seven *hrp* loci in the large cluster of *P.s.* pv. *phaseolicola* is suppressed in complex medium but induced in the plant. Induction occurs in the susceptible host plant as well as in the non-host, tobacco, suggesting that there is no plant species-specific molecule involved in control of host range (RAHME et al. 1992). Five complementation groups, *hrpAB*, *hrpC*, *hrpD*, hrpE and *hrpF*, can also be induced in M9 minimal medium containing sucrose as a carbon source, however, induction is affected by pH, osmolarity, and carbon source, and never reaches the levels obtained in the plant (RAHME et al. 1992). A similar observation was made earlier for the avirulence gene *avrB* in *P.s.* pv. *glycinea*. Induction occurred in a minimal medium containing fructose, mannitol, or sucrose. Expression of *avrB* is dependent on *hrp* genes homologous to *hrpRS* and *hrpL* from *P.s.* pv. *phaseolicola* and was suppressed by TCA cycle intermediates such as citrate and succinate (HUYNH et al. 1989). *hrp* gene expression in *P.s.* pv. *syringae* occurs in the same medium as described by HUYNH et al. (1989); (HUANG et al. 1991; XIAO et al. 1992). The authors showed *hrp* gene induction in the non-host plant, tobacco, but no data for the host plant. The *P.s.* pv. *phaseolicola* loci *hrpL* and *hrpRS* are only expressed to a very low level in M9 minimal medium and are induced at least 1000-fold when the bacteria are inoculated into the plant. This led to the conclusion that, at least for expression of *hrpL* and *hrpRS*, specific plant factors might be necessary (RAHME et al. 1992).

6.2 Regulatory Genes *hrpRS* and *rpoN* of *Pseudomonas syringae* pv. *phaseolicola*

The results on environmental factors inducing or suppressing *hrp* gene expression suggested that specific regulatory genes are involved in the control of *hrp* promoter activities. At least two loci are involved in positiv regulation of the other *hrp* loci of *P.s.* pv. *phaseolicola hrp* cluster (FELLAY et al. 1991). While there is no information published for *hrpL*, *hrpRS* has been sequenced. It contains two genes whose predicted protein products are 60% identical to each other (GRIMM and PANOPOULOS 1989; MILLER et al. 1993). The HrpS protein is similar to members of the NtrC family of regulatory proteins in gram-negative bacteria. Most NtrC-like regulatory proteins are members of two-component systems, with a sensor protein that in turn activates a response element by phosphorylation of a site in the conserved NH_2-terminal domain (ALBRIGHT et al. 1989). The putative sensor component operating in *hrp* gene regulation has not been identified. It is postulated that HrpS is the activating protein, however, direct biochemical data

hav not be n presented. Th lack f a typical NH_2-terminal domain in HrpS could indicate that a different mechanism may be involved in HrpS activation. Apparently, *hrpS*-related sequences are also present in other bacteria, e.g., in *P.s.* pv. *syringae* (Heu and Hutcheson 1993) and in *Erwinia amylovora* (Beer et al. 1993). *E. stewartii* contains a transcriptional regulator, WtsA, with 52% identity to HrpS of *P.s.* pv. *phaseolicola*. The *hrpS* clone, however, was unable to functionally complement a *wtsA* mutant (Frederick et al. 1993).

The structure of the *hrpRS* locus and the finding of –24/–12 consensus sequences upstream of *hrpRS* indicated a possible role in transcriptional activation for transcription factor sigma 54, encoded by *rpoN* (Grimm and Panopoulos 1989). In a preliminary report, Felley et al. (1991) demonstrated that *hrp* gene expression in *P.s.* pv. *phaseolicola* is indeed dependent on *rpoN*. A *rpoN* mutant of *P.s.* pv. *phaseolicola* is a glutamine auxotroph and nonpathogenic. Whether *rpoN* is generally involved in regulation of *hrp* gene expression is not clear. In *X.c.* pv. *vesicatoria*, *rpoN* is clearly not involved in *hrp* gene expression and pathogenicity (T. Horns and U. Bonas, manuscript in preparation).

Recently, Miller et al. (1993) have reported the identification of two new loci, *hrpQ* and *hrpT*, from *P. s.* pv. *phaseolicola* that affect activation of *hrpRS* in *trans*. However, since *hrpRS* is strongly induced in plants while both *hrpQ* and *hrpT* are constitutively expressed, there must be more factors involved in *hrp* gene regulation. Strains carrying mutations in either *hrpQ* or *hrpT* are amino acid auxothrophs (methionine and tryptophan). *hrpQ* and *hrpT* are probably involved in methionine and tryptophan biosynthesis, respectively (Miller et al. 1993). As stated above, such mutants would normally have been eliminated from the *hrp* mutant analysis.

6.3 Conserved Sequence Boxes in *Pseudomonas syringae*

A conserved sequence, the so-called harp box (TG(A/C)AANC, Fellay et al. 1991), upstream of four *hrp* loci in *P. s.* pv. *phaseolicola*, was suggested to be involved in positive regulation of expression. Similar motifs were described for the promoter regions of several *P. syringae* avirulence genes, the expression of which is dependent on *hrpRS* and on *rpoN* (Huynh et al. 1989; Salmeron and Staskawicz 1993; Innes et al. 1993; Shen and Keen 1993). These studies led to a revised 'harp' box sequence (GGAACCNA). Its significance in protein binding has not been shown but *avrD* promoter constructs lacking the harp box are no longer inducible (Shen and Keen 1993). A harp box-related motif was also found upstream of transcripti n unit 3 in *P. solanacearum* (Gough et al. 1993).

There is no harp box sequence in *Xanthomonas hrp* gene promoters. Another sequence motif that occurs in the promoter region of *hrp* loci in *X. c.* pv. *vesicatoria* was recently identified. This "PIP" (plant-inducible promoter) box has the sequence TTCGC-N15-TTCGC and occurs upstream of the –35 consensus sequence in four out of six *hrp* promoters (S. Fenselau and U. Bonas, unpublished). Experiments are underway to test whether this is a protein binding motif.

6.4 *Xanthomonas campestris*

Expression of *hrp* genes in *X. c.* pv. *campestris* was determined after growth in vitro and found to be induced in a minimal medium with sucrose and/or fructose as carbon source. No expression occurred in complex media or with high concentrations of organic nitrogen (Arlat et al. 1991). In *X. c.* pv. *vesicatoria*, expression of the six *hrp* loci is induced in the plant but cannot be efficiently induced in the synthetic media tested so far. However, culture filtrates of sterile tomato cell suspension cultures (called TCM) induced expression of the six *hrp* loci in *X. c.* pv. *vesicatoria* whereas the basal Murashige-Skoog culture medium did not. The inducing factor(s) could only partially be purified from TCM and was found to be smaller than 1000 dalton, heat-stable, organic, and hydrophilic (Schulte and Bonas 1992a). De novo transcription of all *hrp* loci occurs rapidly within 1 h after transfer of the bacteria into TCM (S. Fenselau and U. Bonas, unpublished). A minimal medium was designed which would not suppress *hrp* gene induction. This medium, called XVM1, induces the *hrpF* locus (Fig. 1A) to high levels and differs from the other media described above, in particular by its low concentration in phosphate. Both sucrose and methionine are needed for efficient induction. It is not known whether a plant factor is necessary for activation of the other *hrp* loci, or if the XVM1 medium still lacks components or contains them in suppressing amounts (Schulte and Bonas 1992b).

6.5 *Erwinia* and *Pseudomonas solanacearum*

The *hrp* genes of *Erwinia amylovora* are rapidly induced in the non-host, tobacco, and more slowly in the host, pear. Several loci were induced in minimal medium with mannitol as a carbon source. Induction was suppressed by high concentrations of nitrogen and by glucose and was slightly temperature dependent (Wei et al. 1992b).

In *P. solanacearum*, the *hrp* cluster was also induced in host and in non-host plants, as well as in minimal medium. The best carbon sources for induction of four of the six transcription units were pyruvate and glutamate while, as in other bacteria, casamino acids suppressed induction (Arlat et al. 1992). The two rightmost *hrp* transcription units (5 and 6; Fig. 1B) are constitutively expressed but can be induced under certain conditions (Genin et al. 1992).

The only other gene reported to regulate *hrp* gene expression is *hrpB* from *P. solanacearum*. The gene is part of the *hrp* cluster and appears to be a member of the AraC family of positive regulatory proteins. Interestingly, *hrpB* is related to *virF* of *Yersinia* (Cornelis et al. 1989; Genin et al. 1992). The *hrpB* gene positively regulates four of the six *hrp* loci, as well as the *popA* locus, located outside of the *hrp* cluster which encodes a protein secreted in a Hrp-dependent way (see above; Arlat et al. 1994). Whether the HrpB protein binds directly to *hrp* promoters is not yet known.

At this time one can only speculate whether the regulatory systems for *hrp* gene expression employed by *P. solanacearum* and *P. syringae* are really different

or whether there is a global regulatory network thus allowing the fine tuning of gene expression in response to environmental cues. In conclusion, most *hrp* loci from different bacteria are inducible in a particular minimal medium. At this time it cannot be ruled out that stimulation of *hrp* gene expression involves specific plant factors as was described for the virulence genes of *Agrobacterium* (WINANS 1992). Since the composition of the nutrients available to the pathogen in the plant is not known one can only speculate that the conditions described above reflect the situation in the plant. It is noteworthy that the in vitro culture will only mimic the dynamic nutritional situation that bacteria experience in their interaction with a plant for a short time. In mammalian bacterial pathogens, the expression of genes involved in virulence is also regulated in response to environmental cues rather than to specific host molecules. This subject has been reviewed recently (MEKALANOS 1992 and in accompanying chapters), and I will only mention some important factors. In *Yersinia*, the *vir* and *lcr* genes are regulated by low calcium (low calcium response genes; STRALEY et al. 1993) and by temperature (CORNELIS et al. 1989; see chapter by CORNELIS). A calcium effect has not been described for any plant bacterium. In our laboratory no effect of calcium on *hrpF* gene expression in XVM1 was observed (Schulte and U. Bonas, unpublished). Expression of *invA* of *S. typhimurium* of the *mxi* and *ipa* genes of *Shigella* is affected by osmolarity and the later genes also by temperature (GALÁN and CURTISS 1990; HALE 1991).

Acknowledgements. I thank my previous and present coworkers—Ilse Balbo, Martina Gutschow, Stefan Fenselau, Torsten Horns, Corinne Marie, Michèle Pierre, Ralf Schulte, and Kai Wengelnik— for contributing to the data described here and for fruitful discussions. I am also grateful to my colleagues for sending preprints and sharing unpublished results, and to Heather McKhann and John Mansfield for helpful suggestions on the manuscript. The research in my laboratory was supported in part by grants from the Bundesministerium für Forschung und Technologie (322-4003-0316300A), the Deutsche Forschungsgemeinschaft, and the EEC (BIOT-CT90-0168).

R f rences

Albertini AM, Caramori T, Crabb WD, Scoffone F, Galizzi A (1991) The flaA locus of Bacillus subtilis is part of a large operon coding for flagellar structures, motility functions, and an ATPase-like polypeptide. J Bacteriol 173 3573-3579

Albright LM, Huala E, Ausubel FM (1989) Prokaryotic signal transduction mediated by sensor and regulator protein pairs. Annu Rev Genet 23: 311–336

Allaoui A, Sansonetti PJ, Parsot C (1992) MxiJ, a lipoprotein involved in secretion of Shigella Ipa invasins, is homologous to YscJ, a secretion factor of the Yersinia Yop proteins. J Bacteriol 174: 7661-7669

Allaoui A, Sansonetti PJ, Parsot C (1993) MxiD, an outer membrane protein necessary for the secretion of the Shigella flexneri Ipa invasins. Mol Microbiol 7: 59-68

Andrews GP, Maurelli AT (1992) mxiA of Shigella flexneri 2a, which facilitates export of invasion plasmid antigens, encodes a homolog of the low-calcium response protein, LcrD, of Yersinia pestis. Infect Immun 60: 3287-3295

Arlat M, Gough CL, Barber CE, Boucher C, Daniels MJ (1991) Xanthomonas campestris contains a cluster of hrp genes related to the hrp cluster of Pseudomonas solanacearum. Mol Plant Microbe Interact 4: 593-601

Arlat M, Gough CL, Zischek C, Barberis PA, Trigalet A, Boucher CA (1992) Transcriptional organization and expression of the large hrp gene cluster of Pseudomonas solanacearum. Mol Plant Microbe Interact 5: 187–193

Arlat M, Van Gijsegem F, Huet JC, Pernollet JC, Boucher CA (1994) PopA1, a protein which induces a hypersensitive-like response on specific Petunia genotypes, is secreted via the Hrp pathway of Pseudomonas solanacearum. EMBO J 13: 543–553

Barny MA, Guinebretière MH, Marcais B, Coissac A, Paulin JP, Laurent J (1990) Cloning of a large gene cluster involved in Erwinia amylovora CFBP1430 virulence. Mol Microbiol 4: 777–787

Bauer DW, Beer SV (1991) Further characterization of an hrp gene cluster of Erwinia amylovora. Mol Plant Microbe Interact 4: 493–499

Beer SV, Bauer DW, Jiang X, Laby RJ, Sneath BJ, Wei ZM, Wilcox DA, Zumoff CH (1991) The hrp gene cluster of Erwinia amylovora. In: Hennecke H, Verma DPS (eds) Advances in molecular genetics of plant-microbe interactions, vol 1, Kluwer Academic, Dordrecht, Netherlands, pp 53–60

Beer SV, Wei ZM, Laby RJ, He SY, Bauer DW, Collmer A, Zumoff C (1993) Are harpins universal elicitors of the hypersensitive response of phytopathogenic bacteria? In: Nester EW, Verma DPS (eds) Advances in molecular genetics of plant-microbe interactions vol 2, Kluwer Academic, Dordrecht, Netherlands, pp 281–286

Bischoff DS, Weinreich MR, Ordal GW (1992) Nucleotide sequence of Bacillus subtilis flagellar biosynthetic genes fliP and fliQ and identification of a novel flagellar gene. J Bacteriol 174: 4017–4025

Bonas U, Schulte R, Fenselau S, Minsavage GV, Staskawicz BJ, Stall RE (1991) Isolation of a gene cluster from Xanthomonas campestris pv. vesicatoria that determines pathogenicity and the hypersensitive response on pepper and tomato. Mol Plant Microbe Interact 4: 81–88

Boucher CA, Van Gijsegem F, Barberis PA, Arlat M, Zischek C (1987) Pseudomonas solanacearum genes controlling both pathogenicity and hypersensitivity on tobacco are clustered. J Bacteriol 169: 5626–5632

Boucher CA, Gough CL, Arlat M (1992) Molecular genetics of pathogenicity determinants of Pseudomonas solanacearum with special emphasis on hrp genes. Annu Rev Phytopathol 30: 443–461

Carpenter PB, Ordal GW (1993) Bacillus subtilis FlhA: a flagellar protein related to a new family of signal-transducing receptors. Mol Microbiol 7: 735–743

Coplin DL, Frederick RD, Majerczak DR, Tuttle LD (1992) Characterization of a gene cluster that specifies pathogenicity in Erwinia stewartii. Mol Plant Microbe Interact 5: 81–88

Cornelis G, Sluiters C, Lambert de Rouvroit C, Michiels T (1989) Homology between VirF, the transcriptional activator of the Yersinia virulence regulon, and AraC, the Escherichia coli arabinose operon regulator. J Bacteriol 171: 254–262

Fellay R, Rahme LG, Mindrinos MN, Frederick RD, Pisi A, Panopoulos NJ (1991) Genes and signals controlling the Pseudomonas syringae pv. phaseolicola-plant interaction, in Hennecke H, Verma DPS (eds) Advances in molecular genetics of plant-microbe interactions vol 1. Kluwer Academic, Dordrecht, Netherlands, pp 45–52

Fenselau S, Balbo I, Bonas U (1992) Determinants of pathogenicity in Xanthomonas campestris pv. vesicatoria are related to proteins involved in secretion in bacterial pathogens of animals. Mol Plant-Microbe Interact 4: 593-601

Frederick RD, Majerczak DR, Coplin DL (1993) Erwinia stewartii WtsA, a positive regulator of pathogenicity gene expression, is similar to Pseudomonas syringae pv. phaseolicola HrpS. Mol Microbiol 9: 477–485

Galán JE, Curtiss R III. (1990) Expression of Salmonella typhimurium genes required for invasion is regulated by changes in DNA supercoiling. Infect Immun 58: 1879–1885

Galán JE, Ginocchio C, Costeas P (1992) Molecular and functional characterization of the Salmonella invasion gene invA: homology of InvA to members of a new protein family. J Bacteriol 174: 4338–4349

Genin S, Gough CL, Zischek C, Boucher CA (1992) The hrpB gene encodes a positive regulator of pathogenicity genes from Pseudomonas solanacearum. Mol Microbiol 6: 3065–3076

Genin S, Gough CL, Arlat M, Zischek C, Van Gijsegem F, Barberis P, Boucher CA (1993) Involvement of Pseudomonas solanacearum hrp genes in the secretion of a bacterial compound which induces a hypersensitive-like response on tobacco. In: Nester EW, Verma DPS (eds) Advances in molecular genetics of plant-microbe interactions, vol 2. Kluwer Academic, Dordrecht, Netherlands, pp 259–266

Goguen JD, Yother J, Straley SC (1984) Genetic analysis of the low calcium response in Yersinia pestis Mu d1 (Ap lac) insertion mutants. J Bacteriol 160: 842–848

Gough CL, Genin S, Zischek C, Boucher CA (1992) hrp genes of Pseudomonas solanacearum are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. Mol Plant Microbe Interact 5: 384–389

Gough CL, Genin S, Lopes V, Boucher CA (1993) Homology between the HrpO protein of Pseudomonas

solanacearum and bacterial proteins implicated in a signal peptide independent secretion mechanism. Mol Gen Genet 239: 378–392
Grimm C, Panopoulos NJ (1989) The predicted product of a pathogenicity locus from Pseudomonas syringae pv. phaseolicola is homologous to a highly conserved domain of several prokaryotic regulatory proteins. J Bacteriol 171: 5031–5038
Groisman EA, Ochman H (1993) Cognate gene clusters govern invasion of host epithelial cells by Salmonella typhimurium and Shigella flexneri. EMBO J 12: 3779–3787
Haddix PL, Straley SC (1992) Structure and regulation of the Yersinia pestis yscBCDEF operon. J Bacteriol 174: 4820–4828
Hale TL (1991) Genetic basis for virulence in Shigella species. Microbiol Rev 55: 206–224
He SY, Huang HC, Collmer A (1993) Pseudomonas syringae pv. syringae harpin$_{Pss}$: a protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. Cell 73: 1255–1266
Heu S, Hutcheson SW (1993) Nucleotide sequence and properties of the hrmA locus associated with the Pseudomonas syringae pv. syringae 61 hrp gene cluster. Mol Plant Microbe Interact 6: 553–564
Huang HC, Schuurink R, Denny TP, Atkinson MM, Baker CJ, Yucel I, Hutcheson SW, Collmer A (1988) Molecular cloning of a Pseudomonas syringae pv. syringae gene cluster that enables Pseudomonas fluorescens to elicit the hypersensitive response in tobacco plants. J Bacteriol 170: 4748–4756
Huang Y, Xu P, Sequeira L (1990) A second cluster of genes that specify pathogenicity and host response in Pseudomonas solanacearum. Mol Plant Microbe Interact 3: 48–53
Huang HC, Hutcheson SW, Collmer A (1991) Characterization of the hrp cluster from Pseudomonas syringae pv. syringae 61 and TnphoA tagging of genes encoding exported or membrane-spanning Hrp proteins. Mol Plant Microbe Interact 4: 469–476
Huang HC, He SY, Bauer DW, Collmer A (1992) The Pseudomonas syringae pv. syringae 61 hrpH product, an envelope protein required for elicitation of the hypersensitive response in plants. J Bacteriol 174: 6878–6885
Huang HC, Xiao Y, Lin RH, Lu Y, Hutcheson SW, Collmer A (1993) Characterization of the Pseudomonas syringae pv. syringae hrpJ and hrpI genes: homology of HrpI to a super-family of proteins associated with protein translocation. Mol Plant Microbe Interact 6: 515–520
Huynh TV, Dahlbeck D, Staskawicz BJ (1989) Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. Science 245: 1374–1377
Hwang I, Lim SM, Shaw PD (1992) Cloning and characterization of pathogenicity genes from Xanthomonas campestris pv. glycines. J Bacteriol 174: 1923–1931
Innes RW, Bent AF, Kunkel BN, Bisgrove SR, Staskawicz BJ (1993) Molecular analysis of avirulence gene avrRpt2 and identification of a putative regulatory sequence common to all known Pseudomonas syringae avirulence genes. J Bacteriol 175: 4859–4869
Kamdar HV, Kamoun S, Kado CI (1993) Restoration of pathogenicity of avirulent Xanthomonas oryzae pv. oryzae and X. campestris pathovars by reciprocal complementation with the hrpXo and hrpXc genes and identification of HrpX function by sequence analyses. J Bacteriol 175: 2017–2025
Kamoun S, Kado CI (1990) A plant-inducible gene of Xanthomonas campestris pv. campestris encodes an exocellular component required for growth in the host and hypersensitivity on nonhosts. J Bacteriol 172: 5165–5172
Kamoun S, Kamdar HV, Tola E, Kado CI (1992) Incompatible interactions between crucifers and Xanthomonas campestris involve a vascular hypersensitive response: role of the hrpX locus. Mol Plant Microbe Interact 5: 22–33
Klément Z (1982) Hypersensitivity. In: Mount MS, Lacy GH (eds) Phytopathogenic prokaryotes, vol 2. Academic New York, pp 149–177
Laby RJ, Beer SV (1992) Hybridization and functional complementation of the hrp gene cluster from Erwinia amylovora strain Ea321 with DNA of other bacteria. Mol Plant Microbe Interact 5: 412–419
Lambert de Rouvroit C, Sluiters C, Cornelis GR (1992) Role of the transcriptional activator, VirF, and temperature in the expression of the pYV plasmid genes of Yersinia enterocolitica. Mol Microbiol 6: 395–409
Leite RP, Minsavage GV, Bonas U, Stall RE (1994) Detection and identification of plant pathogenic strains of Xanthomonas based on amplification of DNA sequences related to the hrp genes of Xanthomonas campestris pv. vesicatoria. Appl Environ Microbiol 60: 1068–1077
Lindgren PB, Peet RC, Panopoulos NJH (1986) Gene cluster of Pseudomonas syringae pv. phaseolicola controls pathogenicity on bean plants and hypersensitivity on nonhost plants. J Bacteriol 168: 512–522
Lindgren PB, Panopoulos NJ, Staskawicz BJ, Dahlbeck D (1988) Genes required for pathogenicity and hypersensitivity are conserved and interchangeable among pathovars of Pseudomonas syringae. Mol Gen Genet 211: 499–506

Lindsay WP, Lamb CJ, Dixon RA (1993) Microbial recognition and activation of plant defense systems. Trends Microbiol 1: 181–187
Loubens I, Debarbieux L, Bohin A, Lacroix J-M, Bohin J-P (1993) Homology between a genetic locus (mdoA) involved in the osmoregulated biosynthesis of periplasmid glucans in Escherichia coli and a genetic locus (hrpM) controlling pathologenicity of Pseudomonas syringae. Mol Microbiol 10: 329–340
Mekalanos JJ (1992) Environmental signals controlling expression of virulence determinates in bacteria. J Bacteriol 174: 1–7
Meinhardt LW, Krishnan HB, Balatti PA, Pueppke SG (1993) Molecular cloning and characterization of a sym plasmid locus that regulates cultivar-specific nodulation of soybean by Rhizobium fredii USDA257. Mol Microbiol 9: 17–29
Michiels T, Wattiau P, Brasseur R, Ruysschaert JM, Cornelis G (1990) Secretion of Yop proteins by Yersiniae. Infect Immun 58: 2840–2849
Michiels T, Vanooteghem J-C, Lambert de Rouvroit C, China B, Gustin A, Boudry P, Cornelis GR (1991) Analysis of virC, an operon involved in the secretion of Yop proteins by Yersinia enterocolitica. J Bacteriol 173: 4994–5009
Miller W, Mindrinos MS, Rahme LG, Frederick RD, Grimm C, Gressman R, Kyriakides X, Kokkinidis M, Panopoulos NJ (1993) Pseudomonas syringae pv. phaseolicola-plant interactions: host-pathogen signalling through cascade control of hrp gene expression. In: Nester EW, Verma DPS (eds) Advances in molecular genetics of plant-microbe interactions, vol 2. Kluwer Academic, Dordrecht, pp 267–274
Mukhopadhyay P, Williams J, Mills D (1988) Molecular analysis of a pathogenicity locus in Pseudomonas syringae pv. syringae. J Bacteriol 170: 5479–5488
Mulholland V, Hinton JCD, Sidebotham J, Toth IK, Hyman LJ, Pérombelon MCM, Reeves PJ, Salmond GPC (1993) A pleiotropic reduced virulence (Rvi⁻) mutant of Erwinia carotovora subspecies atroseptica is defective in flagella assembly proteins that are conserved in plant and animal bacterial pathogens. Mol Microbiol 9: 343–356
Neipold F, Anderson D, Mills D (1985) Cloning determinants of pathogenesis from Pseudomonas syringae pv. syringae. Proc Natl Acad Sci USA 82: 406–410
Plano GV, Barve SS, Straley SC (1991) LcrD, a membrane-bound regulator of the Yersinia pestis low-calcium response. J Bacteriol 173: 7293–7303
Rahme LG, Mindrinos MN, Panopoulos NJ (1991) Genetic and transcriptional organization of the hrp cluster of Pseudomonas syringae pv. phaseolicola. J Bacteriol 173: 575–586
Rahme LG, Mindrinos MN, Panopoulos NJ (1992) Plant and environmental sensory signals control the expression of hrp genes in Pseudomonas syringae pv. phaseolicola. J Bacteriol 174: 3499–3507
Ramakrishnan G, Zhao J-L, Newton A (1991) The cell cycle-regulated flagellar gene flbF of Caulobacter crescentus is homologous to a virulence locus (lcrD) of Yersinia pestis. J Bacteriol 173: 7283–7392
Rimpiläinen M, Forsberg A, Wolf-Watz H (1992) A novel protein, LcrQ, involved in the low-calcium response of Yersinia pseudotuberculosis shows extensive homology to YopH. J Bacteriol 174: 3355–3363
Salmeron JM, Staskawicz BJ (1993) Molecular characterization and hrp dependence of the avirulence gene avrPto from Pseudomonas syringae pv. tomato. Mol Gen Genet 239: 6–16
Sanders LA, Van Way S, Mullin DA (1992) characterization of the Caulobacter crescentus flbF promoter and identification of the inferred FlbF product as a homolog of the LcrD protein from a Yersinia enterocolitica virulence plasmid. J Bacteriol 174: 857–866
Saraste M, Gay NJ, Eberle A, Runswick MJ, Walker JE (1981) The atp operon: nucleotide sequences of the genes for the a, b, and g subunits of Escherichia coli ATP synthase. Nucleic Acids Res 9: 5287–5296
Sasakawa C, Komatsu K, Tobe T, Suzuki T, Yoshikawa M (1993) Eight genes in region 5 that form an operon are essential for invasion of epithelial cells by Shigella flexneri. J Bacteriol 175: 2334–2346
Schulte R, Bonas U (1992a) Expression of the Xanthomonas campestris pv. vesicatoria hrp gene cluster, which determines pathogenicity and hypersensitivity on pepper and tomato, is plant inducible. J Bacteriol 174: 815–823
Schulte R, Bonas U (1992b) A Xanthomonas pathogenicity locus is induced by sucrose and sulfur-containing amino acids. Plant Cell 4: 79–86
Shen H, Keen NT (1993) Characterization of the promoter of avirulence gene D from Pseudomonas syringae pv. tomato. J Bacteriol 175: 5916–5924

Stall RE, Minsavage GV (1990) The use of hrp genes to identify opportunistic xanthomonads. In: Klément Z (ed) plant pathogenic bacteria, proceedings of the 7th international conference of plant pathogenic bacteria, Budapest, Hungary, 1989. Akadémiai Kiadó, Budapest, pp 369–374

Steinberger EM, Beer SV (1988) Creation and complementation of pathogenicity mutants of Erwinia amylovora. Mol Plant Microbe Interact 1: 135–144

Straley SC, Plano GV, Skrzypek E, Haddix PL, Fields KA (1993) Regulation by Ca^{2+} in the *Yersinia* low-Ca^{2+} response. Mol Microbiol 8: 1005–1010

Van Gijsegem F, Genin S, Boucher C (1993) Conservation of secretion pathways for pathogenicity determinants of plant and animal bacteria. Trends Microbiol 1: 175–180

Venkatesan MM, Buysse JM, Oaks EV (1992) Surface presentation of Shigella flexneri invasion plasmid antigens requires the products of the spa locus. J Bacteriol 174: 1990–2001

Vogler AP, Homma M, Irikura VM, Macnab RM (1991) Salmonella typhimurium mutants defective in flagellar filament regrowth and sequence similarity of FliI to F0F1, vacuolar, and archaebacterial ATPase subunits. J Bacteriol 173: 3564–3572

Walters K, Maroofi A, Hitchin E, Mansfield J (1990) Gene for pathogenicity and ability to cause hypersensitive reaction cloned from Erwinia amylovora. Physiol Mol Plant Pathol 36: 509–521

Waney VR, Kingsley MT, Gabriel DW (1991) Xanthomonas campestris pv. translucens genes determining host-specific virulence and general virulence on cereals identified by Tn5-gusA insertion mutagenesis. Mol Plant Microbe Interactions 4: 623–627

Wei ZM, Laby RJ, Zumoff CH, Bauer DW, He SY, Collmer A, Beer SV (1992a) Harpin, elicitor of the hypersensitive response produced by the plant pathogen Erwinia amylovora. Science 257: 85-88

Wei ZM, Sneath BJ, Beer SV (1992b) Expression of Erwinia amylovora hrp genes in response to environmental stimuli. J Bacteriol 174: 1875–1882

Wei ZM and Beer SV (1993) HrpI of Erwinia amylovora functions in secretion of harpin and is a member of a new protein family. J Bacteriol 175: 7958–7967

Willis DK, Rich JJ, Hrabak EM (1991) hrp genes of phytopathogenic bacteria. Mol Plant Microbe Interact 4: 132–138

Winans SC (1992) Two-way chemical signaling in Agrobacterium-plant interactions. Microbiol Rev 56: 12–31

Xiao YX, Lu Y, Heu SG, Hutcheson SW (1992) Organization and environmental regulation of the Pseudomonas syringae pv. syringae 61 hrp cluster. J Bacteriol 174: 1734–1741

Th Enigmatic Avirulenc G nes of Phytopathogenic Bacteria

J.L. Dangl

1 Action at the "Pathogenic Cusp"

The previous chapters have discussed how phytopathogenic bacteria can sense and respond to conditions present in a variety of microenvironments: soil, water, plant cell surfaces, and intracellular spaces. The switch from epiphyte t pathogen is apparently accompanied by fundamental reprogramming of gen activity and attendant function, as evidenced by induction of *hrp* genes and subsequent production of various virulence and pathogenicity factors, some of which ar host-specific, some not. This reprogramming switch between epiphytic and pathogenic growth strategies, "the pathogenic cusp" (Dangl 1994), is the point at which not only the potential pathogen but also the host first sense and respond to each other. A successful plant defense response should be based on surveillance and interdiction before the pathogen has a chance to establish production of the armory of factors which determine successful colonization of that host. It is incumbent on each potential plant host, then, to evolve mechanisms to recognize some factor, preferably one produced at this pathog nic cusp, and to base resistance strategies on early recognition. Thus, an evolutionary tug-of-war is

Max-Delbrück-Laboratory, Carl-von-Linné-Weg 10, 50829 Köln, Germany

Journal of Bacteriology, Sept. 1997, p. 5655–5662
0021-9193/97/$04.00+0

Vol. 179, N . 18

MINIREVIEW

The Type III (Hrp) Secretion Pathway of Plant Pathogenic Bacteria: Trafficking Harpins, Avr Proteins, and Death

JAMES R. ALFANO and ALAN COLLMER*

Department of Plant Pathology, Cornell University, Ithaca, New York 14853-4203

INTRODUCTION

The ability of plant pathogenic bacteria to deliver death-triggering proteins to the interior of plant cells was revealed in a rapid succession of papers in 1996 that transformed our concepts of bacterial plant pathogenicity. The breakthrough came with the convergence of work on Hrp systems and Avr pr teins, an understanding of which requires an introduction to the most prevalent bacterial pathogens of plants, the cardinal importance of the Hrp pathway, and the paradoxical phenotype associated with *avr* genes.

Plant pathogenic bacteria in the genera *Erwinia*, *Pseudomonas*, *Xanthomonas*, and *Ralstonia* cause diverse, and sometimes devastating, diseases in many different plants, but they all share three characteristics: they colonize the intercellular spaces of plants, they are capable of killing plant cells, and they possess *hrp* genes. Many of these pathogens are host specific. In host plants, they produce various symptoms after several days of multiplication, whereas in nonhost plants, they trigger the hypersensitive response (HR), a rapid, defense-associated, programmed death of plant cells at the site of invasion (21, 43). With inoculum levels typically encountered in natural environments, the HR produces individual dead plant cells that are scattered within successfully defended healthy tissue (71). However, experimental infiltration of high inoculum levels (>10^6 bacterial cells/ml) results in macroscopically observable death of the entire infiltrated tissue, usually within 24 h (42). Pi neer screens for random transposon mutants with impaired plant interactions yielded a prevalent class that was designated Hrp$^-$, that is, deficient in both HR elicitation in nonhost plant species and pathogenicity (and parasitic growth) in host species (49, 56). This complete loss of pathogenic behavior results from mutation of any one of several *hrp* genes, which largely encode components of a type III protein secretion system (73). Because the capacity to elicit the HR is a convenient marker for the capacity to be pathogenic and these two abilities have a common genetic basis, the "simple" problem of HR elicitation is being studied as an entry to the larger problem of pathogenesis.

A key part of the HR puzzle is that HR elicitation and the resulting limitation in host range can occur if the pathogen possesses any one of many possible *avr* (avirulence) genes that interact with corresponding *R* (resistance) genes in the host plant. Such "gene-for-gene" interactions result in recognition of the bacterium and the triggering of plant defenses. For example, *Pseudomonas syringae* pv. glycinea is one of over 40 *P. syringae* pathovars differing largely in host range among plant species and is subdivided into races on the basis of their interactions with genetically distinct cultivars of its host, soybean. Those race-cultivar interactions involving matching bacterial *avr* and plant *R* genes result in the HR and avirulence, i.e.; failure of the bacterium to produce disease. The *R* genes encode components of a parasite surveillance system and are crossed into crops from wild relatives by plant breeders for disease control. *avr* genes are identified and cloned on the basis of the avirulence they confer on virulent races in appropriate test plants (39, 69). In most cases, it is not clear why plant pathogens carry *avr* genes that betray them to host defenses but new insights into this question are discussed below.

Both *hrp* and *avr* genes were originally defined on the basis of the phenotypes they confer on bacteria interacting with plants. Molecular studies have revealed a functional relationship between the products of these two classes of genes and an underlying similarity with a key virulence system of several animal pathogens. *Yersinia*, *Salmonella*, and *Shigella* spp. transfer virulence effector proteins directly into animal cells via the type III pathway (16, 17, 62, 67, 84). Similarly, plant pathogens use the Hrp type III pathway to transfer Avr effector proteins to the interior of plant cells. The genetic dissection of type III secretion systems is just beginning, and little is known of the mechanisms of protein translocation. In this review, we will describe (i) the recently completed inventory of genes directing type III secretion in plant pathogens and new insights into type III secretion mechanisms gained from research with Hrp systems, (ii) two classes of proteins (harpins and pilins) that are secreted by the Hrp type III pathway when plant pathogens are grown in media that mimic plant intercellular fluids, (iii) evidence that Avr proteins are delivered by the Hrp pathway directly to the interior of plant cells, and (iv) a resulting new paradigm for bacterial plant pathogenicity. The focus will be on quite recent work, and readers are referred to other reviews for a classic introduction to the HR phenomenon (43), earlier investigations of the Hrp system (11), *avr* genes (20, 46), and a wider perspective on bacterial virulence systems and plant responses (2).

Hrp PROTEIN SECRETION SYSTEM

hrp and hrc genes. *hrp* genes have been extensively characterized in four representative gram-negative plant pathogens: *P. syringae* pv. syringae (brown spot of bean), *Erwinia amylovora* (fire blight of apple and pear), *Ralstonia* (*Pseudomonas*) *solanacearum* (bacterial wilt of tomato), and *Xanthomonas campestris* pv. vesicatoria (bacterial spot of pepper and tomato). Most of the known *hrp* genes in these strains are contained in chromosomal clusters of about 25 kb (Fig. 1). In at least some cases, the *hrp* clusters are sufficient to allow HR elicita-

* Corresponding author. Mailing address: Department of Plant Pathology, Cornell University, Ithaca, NY 14853-4203. Phone: (607) 255-7843. Fax: (607) 255-4471. E-mail: arc2@cornell.edu.

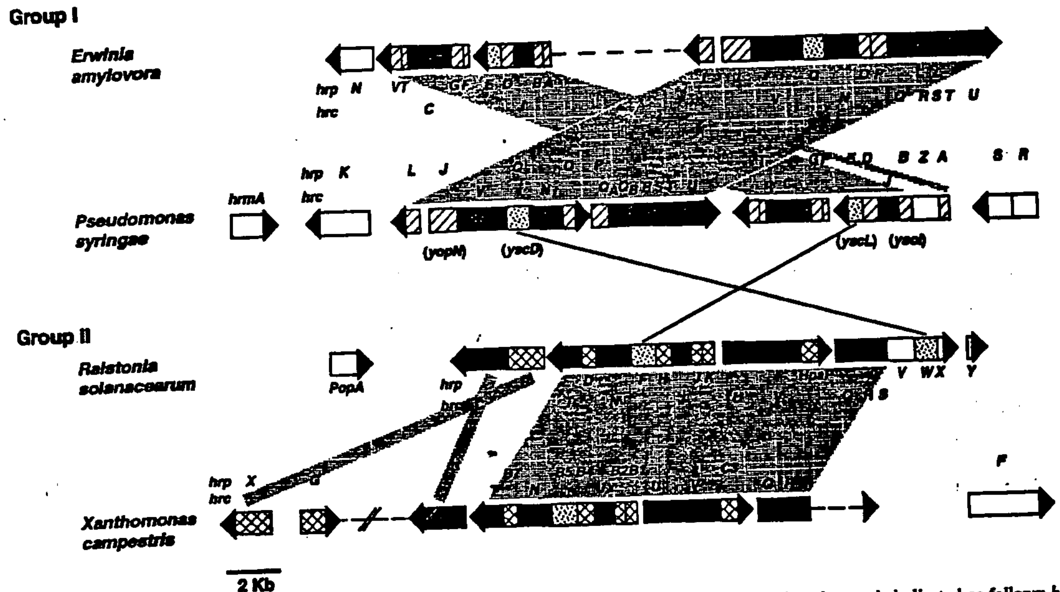


FIG. 1. *hrp* gene clusters of four model plant pathogens. The distribution of each gene among group I and II *hrp* clusters is indicated as follows: *hrc* genes, dark shading; *hrp* genes that are conserved between groups I and II but show weaker similarity to *Yersinia ysc* genes than *hrc* genes, stippling (the two lines between groups indicate homologs); genes common to group I, diagonal lines; genes common to group II, hatching; genes for which no homologs have been reported, white. Dashed lines indicate gaps in the reported sequence of each *hrp* cluster. The shaded bands between members of a group indicate colinear gene arrangements. Note that homologous *hrp* genes have the same designation within group I but not within group II. *Yersinia* genes for which similarity has been noted with *hrp* genes of *R. solanacearum* (74), *E. amylovora* (10, 41), and/or *P. syringae* (36, 60) are in parentheses below the pair of group I *hrp* clusters. The *hrp* cluster of *R. solanacearum* is carried on a megaplasmid (12), but the others appear to be chromosomal. See reference 9 for previous designations of *hrc* genes and for references to all but the recent sequence reports in references 38, 41, and 81.

tion (but not disease) by nonpathogenic bacteria such as *Escherichia coli* and *Pseudomonas fluorescens* (8, 37).

Initial sequencing of the *hrp* clusters from *R. solanacearum*, *X. campestris* pv. vesicatoria, and *P. syringae* pv. syringae revealed homologies with components of the virulence protein (Yop) secretion system of *Yersinia* spp. (22, 29, 34), thereby suggesting the existence of a conserved "type III" protein secretion pathway in gram-negative pathogens of both plants and animals (65, 73). The near completion of these sequences has revealed further homologies and has led to two major changes in the nomenclature of *hrp* genes (9). First, those *hrp* genes that are broadly conserved in pathogenic *Pseudomonas*, *Erwinia*, *Ralstonia*, *Xanthomonas*, *Yersinia*, *Salmonella*, and *Shigella* spp. were redesignated *hrc* (HR and conserved) and given the last-letter designations of their *Yersinia ysc* homologs. The designations for Hrc homologs in various bacteria outside of the plant pathogen group are presented in Table 1. When referred to broadly, the term "*hrp* genes" is intended to encompass the *hrc* subset (9). Second, the *hrp* gene concept was widened to include homologous genes in plant pathogens where mutations do not lead to typical Hrp phenotypes. For example, mutations in *hrp* homologs result in loss of the Wts (watersoaking) phenotype in *Erwinia stewartii* (Stewart's wilt of corn) and reduced infectivity at low inoculum levels in *Erwinia chrysanthemi* (bacterial soft rot) (6, 23). Thus, the *hrp* genes appear to be universal among plant pathogenic *Erwinia*, *Pseudomonas*, *Ralstonia*, and *Xanthomonas* spp. and they control a variety of bacterium-plant interaction phenotypes in addition to the HR.

Group I and II *hrp* clusters. The four *hrp* clusters that have been most characterized can be divided into two groups based on their possession of similar genes, operon structures, and regulatory systems (2). The *hrp* clusters of *P. syringae* and *E. amylovora* are in group I, and those of *R. solanacearum* and *X. campestris* are in group II. In addition to the nine *hrc* genes, two *hrp* genes are conserved between the group I and II *hrp* clusters and show some similarities to *ysc* genes (Fig. 1) (10, 36, 41, 74). It is likely that more of the present *hrp* genes will be discerned as belonging to the *hrc* category with additional data on the structure, function, and conservation of their products in both plant and animal pathogens. Nevertheless, some of the *hrp* genes appear to be completely different between the two groups, the arrangements of genes within some operons are characteristic of each group, and the regulatory systems are distinct (Fig. 1). A key difference in regulation is that group I *hrp* operons are activated by HrpL, a member of the ECF (extracytoplasmic function) subfamily of sigma factors (50, 78, 85), whereas most group II *hrp* operons are activated by a

TABLE 1. Hrc proteins of plant pathogenic bacteria and their animal pathogen and flagellar homologs

Plant pathogen protein[a]	*Yersinia* protein	*Salmonella* protein	*Shigella* protein	Flagellar protein(s)
HrcC	YscC	InvG	MxiD	
HrcJ	YscJ	PrgK	MxiJ	FliF
HrcN	YscN	SpaL	Spa47	FliI
HrcQ	YscQ	SpaO	Spa33	FliN, -Y
HrcR	YscR	SpaP	Spa24	FliP
HrcS	YscS	SpaQ	Spa9	FliQ
HrcT	YscT	SpaR	Spa29	FliR
HrcU	YscU	SpaS	Spa40	FlhB
HrcV	LcrD	InvA	MxiA	FlhA

[a] References for the sequences of *hrc* genes and all homologs are compiled in references 9, 25, and 74.

member of the AraC family, which is designated HrpB in *R. solanacearum* and HrpX in *X. campestris* (27, 57, 82). However, *hrp* genes in both groups are generally repressed in complex media and expressed in plants and in media that mimic plant intercellular fluids (11).

Functions of Hrp and Hrc proteins in type III protein secretion. With the *hrp* clusters of four representative plant pathogens now almost completely sequenced, analysis of the functions of individual components is beginning. Nonpolar mutations have been constructed in most of the *hrp* and *hrc* genes in *R. solanacearum* and in some of the genes in *P. syringae* pv. syringae and *E. amylovora* (10, 15, 54, 77). The results suggest that the secretion apparatus requires all of the *hrc* genes (*hrcQ* awaits testing). The *R. solanacearum* mutant analysis also reveals a requirement for *hrpF*, *-W*, *-K*, and *-X* (54). As discussed above, *hrpF* and *hrpW* have group I and possible *ysc* homologs. Thus, the Hrp type III secretion apparatus is likely composed of a core of 13 proteins, all but 2 of which appear to be broadly conserved. The predicted locations and functions of most of these proteins have been systematically presented for the *R. solanacearum* Hrp system (74), and they appear to be the same in *X. campestris*, *E. amylovora*, and *P. syringae*.

Sequence comparisons reveal that all of the Hrc proteins, other than HrcC, have a homolog involved in flagellum-specific export or early events in flagellum biogenesis (Table 1). The abilities of the presumably more ancient flagellar system to regulate the order (and possibly amount) of protein released and to secrete proteins in association with an extracellular appendage are properties that may be particularly important in the type III transfer of virulence proteins into host cells (18, 52). Plant pathogens offer several experimental advantages for exploring mechanisms of type III secretion and, indirectly, flagellum-specific secretion. The flagellum-specific and animal pathogen type III secretion systems have been difficult to study because many mutations pleiotropically disrupt production of the secretion apparatus and the secreted proteins. For example, the *Yersinia pestis* LcrD and *Bacillus subtilis* FlhA proteins (homologs of HrcV) were initially thought to have primary functions in regulation (14, 59). However, the unambiguous secretion phenotype of an *E. amylovora hrcV* mutant provided strong evidence that the primary function of members of this protein superfamily is in secretion (77). Plant pathogens offer other experimental advantages for exploring type III secretion mechanisms: defined subclones of ca. 25 kb are conveniently sufficient for Hrp-mediated secretion by *E. coli* and other model bacteria (31, 77), and *hrc* gene arrangements and mutant phenotypes suggest that translocation across the inner and outer membranes is partially separable in these bacteria (15).

In both group I and II *hrp* clusters, the six *hrc* genes predicted to encode a flagellum-derived system for Sec-independent translocation across the inner membrane (*hrcN*, *-R*, *-S*, *-T*, *-U*, and *-V*) are in operons other than that containing the one *hrc* gene predicted to direct translocation across the outer membrane (*hrcC*) (Fig. 1 and 2). HrcC is a member of the PulD/pIV superfamily of outer membrane proteins, which are involved in type II protein secretion (PulD) and filamentous phage secretion (pIV) (26). These proteins form homomultimers in the outer membrane which permit phage or protein exit and induce the *psp* (phage shock protein) operon (63). The HrcC protein of *X. campestris* pv. vesicatoria was the first member of the type III branch of this superfamily shown to induce the *psp* operon, thereby suggesting that the type III pathway also employs an outer membrane, channel-forming multimer (80). A *P. syringae* pv. syringae *hrcC* mutant accumulates some of the normally secreted HrpZ harpin (discussed below) in the periplasm, whereas a *hrcU* mutant accumulates

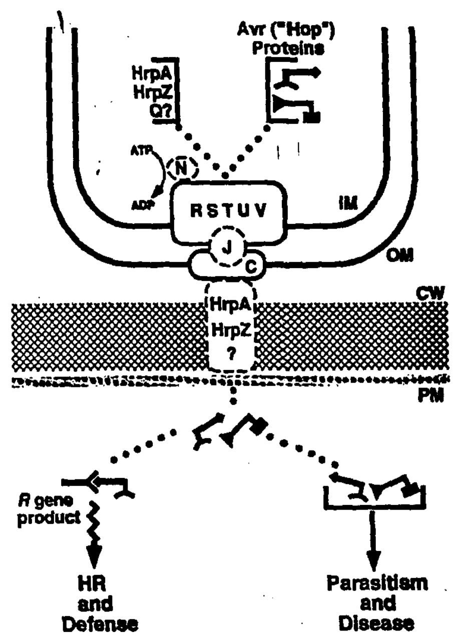


FIG. 2. Model for the delivery of parasite-promoting Avr proteins (i.e., Hop proteins according to a proposal discussed in the text) into plant cells by the Hrp type III secretion system (*P. syringae* example). To reach their targets, Avr proteins must cross the bacterial inner membrane (IM), outer membrane (OM), plant cell wall (CW), and plasma membrane (PM). Only Hrc components (indicated by their last letters in predicted subcellular locations) and proteins known to be secreted are shown. The location of hydrophilic HrcQ ($HrcQ_A$ and $HrcQ_B$ in *P. syringae*) is unknown, but the homologous SpaO is secreted by *Salmonella* spp. (25, 48). Four additional Hrp proteins, not shown, appear to be required for secretion (see text). Dashed-line boxes indicate uncertainties about precise location. For example, it is not known whether HrpA or HrpZ penetrates the plant cell wall and whether these and/or other Hrp proteins trigger Avr transfer into plant cells by endocytosis. Secretion of HrpA and HrpZ is not dependent on plant cell contact, whereas secretion of Avr proteins apparently is. Once inside plant cells, multiple Avr proteins apparently promote parasitism collectively by unknown mechanisms (short arms denote weak phenotypes of virulence domains interacting with undefined host targets), unless any one of the proteins interacts with a host *R* gene product, thereby triggering the HR defense. Mutation of a host target, to diminish benefit to the parasite, and detection by the *R* gene surveillance system are likely evolutionary responses of plants to the bacterial deployment of a new virulence protein; coevolution would be expected to generate many *avr* and *R* genes in complex populations of plants and bacterial parasites.

the protein exclusively in the cytoplasm (15). Thus, the sequence-based prediction that separate inner and outer membrane translocator systems have been recruited to form the Hrp pathway is supported by a novel secretion phenotype revealing partial separation of these functions (15).

HARPINS, PILINS, AND OTHER PROTEINS SECRETED IN CULTURE BY THE Hrp SYSTEM

Harpins. Broadly defined, harpins are glycine-rich proteins that lack cysteine, are secreted in culture when the Hrp system is expressed, and possess heat-stable HR elicitor activity when they infiltrate the leaves of tobacco and several other plants. As is characteristic of proteins secreted by the type III pathway, harpins lack an N-terminal signal peptide. The first harpin was discovered in the culture fluids of *E. coli* cells carrying a highly expressed *hrp* cluster from *E. amylovora* (79). Because mutations in the harpin-encoding *hrpN* gene in *E. amylovora* strongly diminish HR elicitation in tobacco and pathogenicity in susceptible, immature pear fruits, harpin was initially thought to be the primary virulence protein traveling the Hrp pathway

(79). Subsequent analysis of harpins from other bacteria has revealed that harpins differ substantially in their primary structure and their contribution to Hrp phenotypes, and their actual function is unknown (4, 7, 19, 31).

The harpin genes of *E. amylovora* (*hrpN*) (79), *E. chrysanthemi* (*hrpN$_{Ech}$*) (7), and *R. solanacearum* (*popA*) (4) are located adjacent to or near their respective *hrp* clusters, whereas the *P. syringae hrpZ* gene resides within a *hrp* operon (31). *E. chrysanthemi hrpN* mutants are reduced in infectivity at low inoculum levels and are unable to elicit the HR (7), but harpin gene mutations in *E. amylovora* CFBP1430 (a highly virulent strain) (5), *R. solanacearum* (4), and *P. syringae* (1) produce weak phenotypes or no phenotype. Thus, individual harpins do not appear to be necessary for elicitation of the HR by most bacteria. The potential role of harpins in determining host specificity is uncertain. PopA may be a host specificity factor because the isolated protein elicits the HR selectively in those plants in which *R. solanacearum* also elicits the HR, whereas the isolated harpins from *E. amylovora* and three *P. syringae* pathovars trigger the HR in various plants in a manner that shows no relationship to bacterial host range (30, 31, 60, 79). Harpin activity may involve interactions with plant cell walls. The HrpZ harpin binds to the walls of intact plant cells but not to protoplasts, and it also fails to trigger HR-associated responses in protoplasts (33). The elicitor activity of harpins is unlikely to be enzymatically based because various fragments retain activity (1, 4, 45).

The function of the *P. syringae* HrpZ harpin is particularly puzzling. Several observations suggest a simple, direct role for HrpZ in HR elicitation. HrpZ is the predominant protein secreted by the *P. syringae* Hrp system in culture (31, 88), the *hrpZ* gene is conserved in divergent *P. syringae* pathovars (60), and the isolated protein elicits an apparent programmed cell death in plants that is indistinguishable from the HR elicited by living bacteria (31). Furthermore, *hrpZ* deletion mutations in the cosmid pHIR11 functional cluster of *P. syringae* pv. syringae *hrp* genes strongly reduce the HR elicitation activity of *E. coli* cells carrying only pHIR11. The same mutation only slightly reduces the HR in *P. syringae* pv. syringae, but this can be explained by postulating the existence of a second harpin encoded elsewhere in the bacterial genome (1).

However, other observations show that the relationship of HrpZ to HR elicitation is more complex. Mutation of *hrmA* (32, 35), which is in a variable region flanking the conserved *hrp* cluster in pHIR11, abolishes HR activity in tobacco without diminishing HrpZ synthesis or secretion (1). Thus, isolated HrpZ is sufficient to elicit an HR in tobacco leaves but HrpZ produced by bacteria in plants is not. Instead, HrmA, with no apparent function in the Hrp secretion apparatus, is necessary for bacterial elicitation of the HR, and thus, HrmA appears to be the actual elicitor of the HR produced by bacteria carrying pHIR11. HrmA has several characteristics of an Avr protein (3). Avr proteins and the role of the Hrp system (and possibly harpins) in their delivery into plant cells will be discussed below.

HrpA pilin and other secreted proteins. *P. syringae* pv. tomato DC3000 secretes at least four proteins in addition to HrpZ into the medium in a Hrp-dependent manner (88). One of these is the 10-kDa product of *hrpA*, which forms a 6- to 8-nm-diameter "Hrp pilus" (61). A nonpolar *hrpA* mutant no longer elicits the HR in appropriate test plants, even when carrying an *avr* gene known to interact with an *R* gene in the plant. It thus appears that the Hrp pilus is essential for the delivery of Avr signals (discussed further below). Although it is not known whether the Hrp pilus functions primarily in bacterial attachment or as a conduit for the delivery of bacterial proteins across the plant cell wall, it is interesting that *Agrobacterium tumefaciens* requires a pilus similar in size (3.8-nm diameter) to transfer T-DNA and the VirE2 protein into plant cells (24).

Hrp DELIVERY OF AVR PROTEINS INTO PLANT CELLS

***avr* genes and their products.** In fundamental contrast to the *hrp* genes, *avr* genes are scattered in their distribution among strains of plant pathogenic bacteria (20, 46). More than 30 bacterial *avr* genes have been cloned from *P. syringae* and *X. campestris*, but until recently, characterization of the menagerie of encoded proteins has largely defined what these proteins do not do. Isolated Avr proteins do not elicit any responses when they infiltrate plant leaves. They do not appear to be secreted in culture and are hydrophilic proteins lacking N-terminal signal peptides or other recognizable secretion signals (properties consistent with potential secretion by the type III pathway). They do not have demonstrable enzymatic activity (with the exception of AvrD, which directs the synthesis of syringolide elicitors of an *R* gene-dependent HR [55]), and the majority of them do not contribute in an obvious way to parasitic fitness or virulence in the infection of cultivars lacking a matching *R* gene that would trigger the HR. However, there are several significant exceptions to the last point (20, 46) and there is growing evidence that Avr proteins have a primary function in virulence, even though the HR-triggering effects of *Avr-R* interactions are epistatic over these virulence functions. How Avr proteins might promote parasitism is mysterious, but support for such a primary role comes from observations that their action is dependent on the Hrp system and their site of action is within host cells. The next two sections address these points and provide evidence that the main function of the Hrp system is in the delivery of Avr-like proteins into plant cells.

Hrp dependency of Avr phenotypes. *avr* genes have no phenotype when expressed in *hrp* mutant pathogens or in nonpathogenic bacteria like *E. coli*, which lack the Hrp system (highly expressed *avrD* is the sole exception to the latter point [40]). For many *avr* genes, especially those in *P. syringae*, one simple explanation is that their expression is dependent on Hrp regulatory factors (46). However, expression of *avr* genes from vector promoters does not obviate the requirement for a functional Hrp system. The recent finding that the functional cluster of *P. syringae* pv. syringae *hrp* genes carried on cosmid pHIR11 is sufficient to deliver heterologous *avr* gene signals indicates the fundamental interdependency of Hrp and Avr functions in bacterial elicitation of the HR (28, 58). A key property of pHIR11 enabling this discovery is that the cosmid confers on nonpathogenic *E. coli* and *P. fluorescens* the ability to elicit the HR in tobacco and several other plants, but it is ineffective in doing so in soybean and *Arabidopsis*. The simplest explanation is that *hrmA*, which is carried on pHIR11 and has several properties of *avr* genes (3), interacts with an unknown *R* gene in tobacco but with no *R* genes in soybean and *Arabidopsis*. This suggested that expression of appropriate *avr* genes in *trans* would enable nonpathogens carrying pHIR11 to elicit an *R* gene-dependent HR in soybean, *Arabidopsis*, and other plants. Indeed, this was observed with *avrB* (from *P. syringae* pv. glycinea) and five other *P. syringae avr* genes (28, 58).

The ability of pHIR11 to deliver *avr* gene signals requires HrcC (absolutely) and HrpZ (variably) (28, 58). The inability of HrpZ to support AvrB signal delivery when supplied exogenously indicates that the harpin has a role only when produced along with AvrB and therefore may be an extracellular accessory in the delivery of Avr proteins, as YopD is in the

delivery of YopE (28, 62). Most importantly, these experiments reveal that a functional Hrp secretion system is required for the delivery of several *avr* gene signals. Furthermore, the use of promoters different in strength and of epitope-tagged AvrB revealed that the requirement for a functional Hrp secretion system cannot be obviated by high levels of AvrB in the bacterial cytoplasm or by infiltration of leaves with purified AvrB at a level 1,000-fold higher than that required by living Hrp^+ bacteria to elicit the HR (28). Thus, AvrB does not appear to act in the bacterial cytoplasm or in leaf intercellular spaces. These observations strongly support the hypothesis, depicted in Fig. 2, that the type III protein secretion system in plant pathogens, as in animal pathogens, is capable of delivering bacterial proteins into host cells.

Demonstrations of Avr action in host cells. Bacterial transfer of Avr proteins into plant cells has not been observed directly. However, there is evidence that several of these proteins are biologically active when produced within plant cells, that the HR-triggering activity of one of them is dependent on physical interaction with its cognate plant *R* gene product, and that the activity of another is dependent on localization to the plant cell nucleus. AvrB action in plant cells was demonstrated with *Arabidopsis* plants carrying the cognate *RPM1 R* gene (28). An *Arabidopsis rpm1* mutant was transformed with *avrB* and crossed with a wild-type line, thus producing seedling progeny carrying both *avrB* and *RPM1* which died soon after germinating. One symptomless *rpm1* mutant transgenic plant was obtained; this individual expressed relatively low levels of an *avrB* construct carrying the PR-1a plant protein signal peptide, with the likely consequence that the plant cytoplasm would be exposed only transiently or to low levels of AvrB. The properties of this survivor suggest that plants are sensitive to AvrB even in the absence of a functional matching *R* gene and that vanishingly low levels of the protein are sufficient to elicit the HR in the presence of a complete *R* gene. A biolistic, transient expression assay revealed that *avrB* lacking a signal peptide (and therefore localized to the plant cytoplasm) was lethal to *Arabidopsis* leaf cells carrying *RPM1* but not to those lacking the *R* gene (28). This approach was extended with *avrRpt2* (from *P. syringae* pv. tomato) (47). Similarly, an *A. tumefaciens* transient expression system was used to deliver *avrPto* (from *P. syringae* pv. tomato) and *avrBs3* (from *X. campestris* pv. vesicatoria) into plants, resulting in an *R* gene-dependent HR in all cases (66, 70, 72). Thus, whereas no bacterial Avr protein has been observed to have an effect when delivered exclusively to the surface of plant cells, all four of those tested elicit an *R* gene-dependent response when expressed inside them.

The simplest model for the molecular basis of gene-for-gene HR elicitation predicts physical interaction between the protein products of cognate *avr* and *R* genes. This has been observed with the bacterial AvrPto and plant Pto proteins; mutations in the molecular partners that diminish physical interaction in the yeast two-hybrid system also diminish biological function (66, 70). Because AvrPto action requires a functional Hrp system in either *P. syringae* pv. tomato (64) or nonpathogens carrying the pHIR11 *hrp* cluster (28, 58) and it involves physical interaction with a cytoplasmic target in the host, the Hrp-mediated transfer of AvrPto into plant cells seems certain.

While many bacterial Avr proteins appear to b targeted to the host plant cytoplasm, members of th AvrBs3 family in *Xanthomonas* spp. are targeted to the host nucleus. These proteins carry functional nuclear localization signals (NLS) in the C-terminal region (72, 86). When fusions of this C-terminal region and a *uidA* reporter are transiently expressed in onion epidermal cells by biolistic bombardment, β-glucuronidase activity is localized to the nucleus (72, 86). Deletion of all three of the NLS sequences abolishes nuclear localization in the biolistics assay and HR elicitation by *X. campestris* pv. vesicatoria cells in pepper plants carrying the *Bs3 R* gene, and both of these abilities can be restored by substitution of the simian virus 40 large-T antigen NLS (72). These results suggest that the *Bs3* product must also be localized to the nucleus, but because this *R* gene has not been cloned, this awaits confirmation.

Gaps in our knowledge of the Hrp pathway and the inventory of its protein traffic. Although the rings of evidence that the Hrp system transfers Avr proteins into plant cells are collectively strong, there are formal gaps in each. (i) In the system explored in the most detail, AvrPto-Pto, physical interaction between the bacterial and plant proteins has not been demonstrated in vivo, and a second host protein, Prf, is required for AvrPto-Pto-mediated HR elicitation. Furthermore, all of the other cloned plant *R* genes that interact with known bacterial *avr* genes resemble Prf (a nucleotide-binding site leucine-rich repeat protein) rather than Pto (a kinase) (68). (ii) R proteins appear to be present at vanishingly low levels, and none has been directly observed in the cytoplasm, although RPS2 localizes to the cytoplasm-equivalent fraction in a rabbit reticulocyte dog pancreatic microsome in vitro translation-translocation system (47). (iii) Similarly, Avr proteins appear to be effective at vanishingly low levels (28) and immunogold labeling and electron microscopy of infected plant tissues has revealed their presence only in bacterial cells (13, 87). (iv) Finally, no Avr protein has been directly shown to be translocated out of the bacterial cytoplasm in culture by the Hrp system. It is worth noting that the *A. tumefaciens* VirE2 protein has never been observed to be transferred into plant cells, although the indirect evidence for its action within plant cells seems irrefutable (89).

Many (if not most) of the genes encoding proteins that are transferred into plant cells by these bacterial pathogens probably await discovery. Systematic completion of the inventory is thwarted by two problems. First, the contribution of the genes to virulent interactions may be too subtle for detection in mutant screens, and cognate *R* genes that would reveal Avr phenotypes when the bacterial genes are heterologously expressed may be unknown or nonexistent. Second, no plant signals or regulatory mutants have been found that permit bacteria to secrete these proteins in culture, although harpins, pilins, and possibly other proteins that serve the type III secretion system are secreted in culture. A critical feature of the type III protein secretion system in *Yersinia* spp. is its capacity to withhold full secretion of virulence proteins until contact with the host cell (18). The fact that nonpathogens carrying the pHIR11 functional *hrp* cluster secrete HrpZ but not AvrB in culture (28) indicates that the genetic information for this expected regulatory step is carried within the *hrp* cluster and is therefore subject to discovery through systematic analysis of the *hrp* genes. Obtaining Avr protein secretion in culture is important because (i) it is likely to be associated with structures that normally are used to penetrate the plant cell wall (and possibly trigger host cell endocytosis) and therefore will yield clues to the transfer process and (ii) it will allow proteins targeted to the host to be systematically characterized through identificati n of novel proteins in the medium. The exploration of DNA sequences flanking *hrp* clusters also should be useful in this search because of the growing evidence that these regions are enriched in genes whose products probably travel the Hrp pathway (51, 53, 54).

A new designation for effector proteins that are delivered by the Hrp system to plant cells would be useful: Avr appears

to be inappropriate because some of the encoding genes may have no Avr phenotype and the primary function of Avr proteins is almost certainly in virulence, not avirulence. One proposal is to designate new members of this class Hop (Hrp-dependent outer protein) and to add a four-letter suffix identifying the bacterial species, pathovar, and gene, based on the current system for uniform nomenclature of avirulence genes (3, 75). For example, the gene encoding a newly found *P. syringae* pv. syringae protein in this class would be designated *hopPsyA*. Hop is analogous to the Yop (Yersinia outer protein) designation for proteins secreted by the prototypical *Yersinia* type III secretion system but is broadened here for consistency with the use of Hrp and Avr for plant pathogens in all genera.

A NEW PARADIGM AND FUTURE EXPLORATIONS

Pathogenesis based on the Hrp delivery of Avr-like (Hop) proteins into host cells (depicted in Fig. 2) provides a simple and unifying explanation for many characteristics of plant pathogenic *Erwinia*, *Pseudomonas*, *Xanthomonas*, and *Ralstonia* spp. (2). These include the one-to-one relationship between bacterial cells and HR-responding plant cells (expected with contact-dependent secretion), the gene-for-gene interactions of pathogen races and host cultivars (expected if *avr* and *R* gene products can directly interact within host cells), and the enormous diversity in host range and other pathogenic attributes among closely related strains (expected with a pool of horizontally transferable and interchangeable genes whose products can either promote or betray parasites in coevolving hosts). The latter point is particularly relevant to *P. syringae* and *X. campestris*, which are divided into more than 40 and 140 pathovars, respectively. And it is consistent with the location of many *avr* genes on plasmids and the ability of *avr* genes to function with heterologous Hrp systems (20). In this regard, one potential difference between the type III systems of animal and plant pathogens is noteworthy. In animal pathogen type III systems, the secretion of many effector proteins requires customized chaperones, which are often encoded by genes linked to effector genes (76). The ability of many isolated *avr* genes to function heterologously in other pathogens or in nonpathogens carrying the pHIR11 functional *hrp* cluster suggests that Avr protein delivery does not require specific chaperones or that a promiscuous chaperone gene exists within the *hrp* cluster.

This new model of plant pathogenicity invites several fundamental questions in plant pathology and pathogenic microbiology in addition to those discussed above regarding the Hrp system and the identification of its traffic. How do Hrp-delivered proteins alter host metabolism to promote bacterial growth in plant intercellular spaces? How is host specificity determined at the pathovar-host species level? That is, are *avr-R* gene interactions important here also, as suggested by the discovery of novel *avr* genes through expression in heterologous pathovars (44, 83), or do Avr-like proteins have important positive effects in bacterial adaptation to host species? Given the use of homologous secretion systems, how similar are the functions of the virulence proteins that plant and animal pathogens transfer into their hosts? Sequence similarities involving secreted *Yersinia* proteins have been noted only between YopN and YopJ and the *E. amylovora* HrpJ and *X. campestris* pv. vesicatoria AvrRxv proteins, respectively (10, 46). Since YopN appears to be an extracellular component of the secretion system and the effector activity of YopJ is unknown, this key question remains unanswered. Further comparisons should give us a broader perspective on the evolution of bacterial pathogenicity and may lead to unanticipated controls for diseases of both plants and animals.

ACKNOWLEDGMENTS

We thank Gail Preston, Amy O. Charkowski, Jong Hyun Ham, David W. Bauer, Adam J. Bogdanove, Jihyun F. Kim, and Steven V. Beer for helpful comments and Christian Boucher, Ulla Bonas, and Steven V. Beer for providing manuscripts prior to publication.

Work in the authors' laboratory is supported by grants from the National Science Foundation (MCB 9305178) and the National Research Initiative Competitive Grants Program/U.S. Department of Agriculture (94-37303-0734).

REFERENCES

1. Alfano, J. R., D. W. Bauer, T. M. Milos, and A. Collmer. 1996. Analysis of the role of the *Pseudomonas syringae* pv. *syringae* HrpZ harpin in elicitation of the hypersensitive response in tobacco using functionally nonpolar deletion mutations, truncated HrpZ fragments, and *hrmA* mutations. Mol. Microbiol. 19:715–728.
2. Alfano, J. R., and A. Collmer. 1996. Bacterial pathogens in plants: life up against the wall. Plant Cell 8:1683–1698.
3. Alfano, J. R., H.-S. Kim, T. P. Delaney, and A. Collmer. 1997. Evidence that the *Pseudomonas syringae* pv. *syringae hrp*-linked *hrmA* gene encodes an Avr-like protein that acts in a *hrp*-dependent manner within tobacco cells. Mol. Plant-Microbe Interact. 10:580–588.
4. Arlat, M., F. Van Gijsegem, J. C. Huet, J. C. Pernollet, and C. A. Boucher. 1994. PopA1, a protein which induces a hypersensitive-like response on specific *Petunia* genotypes, is secreted via the Hrp pathway of *Pseudomonas solanacearum*. EMBO J. 13:543–553.
5. Barny, M.-A. 1995. *Erwinia amylovora hrpN* mutants, blocked in harpin synthesis, express a reduced virulence on host plants and elicit variable hypersensitive reactions on tobacco. Eur. J. Plant Pathol. 101:333–340.
6. Bauer, D. W., A. J. Bogdanove, S. V. Beer, and A. Collmer. 1994. *Erwinia chrysanthemi hrp* genes and their involvement in soft rot pathogenesis and elicitation of the hypersensitive response. Mol. Plant-Microbe Interact. 7: 573–581.
7. Bauer, D. W., Z.-M. Wei, S. V. Beer, and A. Collmer. 1995. *Erwinia chrysanthemi* harpin$_{Ech}$: an elicitor of the hypersensitive response that contributes to soft-rot pathogenesis. Mol. Plant-Microbe Interact. 8:484–491.
8. Beer, S. V., D. W. Bauer, X. H. Jiang, R. J. Laby, B. J. Sneath, Z.-M. Wei, D. A. Wilcox, and C. H. Zumoff. 1991. The *hrp* gene cluster of *Erwinia amylovora*, p. 53–60. *In* H. Hennecke and D. P. S. Verma (ed.), Advances in molecular genetics of plant-microbe interactions. Kluwer Academic Publishers, Dordrecht, The Netherlands.
9. Bogdanove, A. J., S. V. Beer, U. Bonas, C. A. Boucher, A. Collmer, D. L. Coplin, G. R. Cornelis, H.-C. Huang, S. W. Hutcheson, N. J. Panopoulos, and F. Van Gijsegem. 1996. Unified nomenclature for broadly conserved *hrp* genes of phytopathogenic bacteria. Mol. Microbiol. 20:681–683.
10. Bogdanove, A. J., Z.-M. Wei, L. Zhao, and S. V. Beer. 1996. *Erwinia amylovora* secretes harpin via a type III pathway and contains a homolog of *yopN* of *Yersinia* spp. J. Bacteriol. 178:1720–1730.
11. Bonas, U. 1994. *hrp* genes of phytopathogenic bacteria. Curr. Top. Microbiol. Immunol. 192:79–98.
12. Boucher, C. A., A. Martinel, P. Barberis, G. Alloing, and C. Zischeck. 1986. Virulence genes are carried by a megaplasmid of the plant pathogen *Pseudomonas solanacearum*. Mol. Gen. Genet. 205:270–275.
13. Brown, I., J. Mansfield, I. Irlam, J. Conrads-Strauch, and U. Bonas. 1993. Ultrastructure of interactions between *Xanthomonas campestris* pv. *vesicatoria* and pepper, including immunocytochemical localization of extracellular polysaccharides and the AvrBs3 protein. Mol. Plant-Microbe Interact. 6:376–386.
14. Carpenter, P. B., and G. W. Ordal. 1993. *Bacillus subtilis* FlhA: a flagellar protein related to a new family of signal-transducing receptors. Mol. Microbiol. 7:735–743.
15. Charkowski, A. O., H.-C. Huang, and A. Collmer. 1997. Altered localization of HrpZ in *Pseudomonas syringae* pv. syringae *hrp* mutants suggests that different components of the type III secretion pathway control protein translocation across the inner and outer membranes of gram-negative bacteria. J. Bacteriol. 179:3866–3874.
16. Chen, Y., M. R. Smith, K. Thirumalai, and A. Zychlinsky. 1996. A bacterial invasion induces macrophage apoptosis by binding directly to ICE. EMBO J. 15:3853–3860.
17. Collazo, C. M., and J. E. Galán. 1997. The invasion-associated type III system of *Salmonella typhimurium* directs the translocation of Sip proteins into the host cell. Mol. Microbiol. 24:747–756.
18. Cornelis, G. R., and H. Wolf-Watz. 1997. The *Yersinia* Yop regulon: a bacterial system for subverting eukaryotic cells. Mol. Microbiol. 23:861–867.
19. Cui, Y., L. Madi, A. Mukherjee, C. K. Dumenyo, and A. K. Chatterjee. 1996. The RsmA$^-$ mutants of *Erwinia carotovora* subsp. *carotovora* strain Ecc71 overexpress *hrpN$_{Ecc}$* and elicit a hypersensitive reaction-like response in tobacco leaves. Mol. Plant-Microbe Interact. 9:565–573.
20. Dangl, J. L. 1994. The enigmatic avirulence genes of phytopathogenic bacteria. Curr. Top. Microbiol. Immunol. 192:99–118.

HR-def. 21. Dangl, J. L., R. A. Dietrich, and M. H. Richberg. 1996. Death don't have no mercy: cell death programs in plant-microbe interactions. Plant Cell 8:1793–1807.
22. Fenselau, S., I. Balbo, and U. Bonas. 1992. Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. Mol. Plant-Microbe Interact. 5:390–396.
23. Frederick, R. D., D. R. Majerczak, and D. L. Coplin. 1993. *Erwinia stewartii* WtsA, a positive regulator of pathogenicity gene expression, is similar to *Pseudomonas syringae* pv. *phaseolicola* HrpS. Mol. Microbiol. 9:477–485.
24. Fullner, K. J., J. C. Lara, and E. W. Nester. 1996. Pilus assembly by *Agrobacterium* T-DNA transfer genes. Science 273:1107–1109.
25. Galán, J. E. 1996. Molecular genetic bases of *Salmonella* entry into host cells. Mol. Microbiol. 20:263–271.
26. Genin, S., and C. A. Boucher. 1994. A superfamily of proteins involved in different secretion pathways in gram-negative bacteria: modular structure and specificity of the N-terminal domain. Mol. Gen. Genet. 243:112–118.
27. Genin, S., C. L. Gough, C. Zischek, and C. A. Boucher. 1992. Evidence that the *hrpB* gene encodes a positive regulator of pathogenicity genes from *Pseudomonas solanacearum*. Mol. Microbiol. 6:3065–3076.
28. Gopalan, S., D. W. Bauer, J. R. Alfano, A. O. Loniello, S. Y. He, and A. Collmer. 1996. Expression of the *Pseudomonas syringae* avirulence protein AvrB in plant cells alleviates its dependence on the hypersensitive response and pathogenicity (Hrp) secretion system in eliciting genotype-specific hypersensitive cell death. Plant Cell 8:1095–1105.
29. Gough, C. L., S. Genin, C. Zischek, and C. A. Boucher. 1992. *hrp* genes of *Pseudomonas solanacearum* are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. Mol. Plant-Microbe Interact. 5:384–389.
30. He, S. Y., D. W. Bauer, A. Collmer, and S. V. Beer. 1994. Hypersensitive response elicited by *Erwinia amylovora* harpin requires active plant metabolism. Mol. Plant-Microbe Interact. 7:289–292.
31. He, S. Y., H.-C. Huang, and A. Collmer. 1993. *Pseudomonas syringae* pv. *syringae* harpin$_{Pss}$: a protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. Cell 73:1255–1266.
32. Heu, S., and S. W. Hutcheson. 1993. Nucleotide sequence and properties of the *hrmA* locus associated with the *Pseudomonas syringae* pv. *syringae* 61 *hrp* gene cluster. Mol. Plant-Microbe Interact. 6:553–564.
33. Hoyos, M. E., C. M. Stanley, S. Y. He, S. Pike, X.-A. Pu, and A. Novacky. 1996. The interaction of Harpin$_{Pss}$ with plant cell walls. Mol. Plant-Microbe Interact. 9:608–616.
34. Huang, H.-C., S. Y. He, D. W. Bauer, and A. Collmer. 1992. The *Pseudomonas syringae* pv. syringae 61 *hrpH* product, an envelope protein required for elicitation of the hypersensitive response in plants. J. Bacteriol. 174:6878–6885.
35. Huang, H.-C., S. W. Hutcheson, and A. Collmer. 1991. Characterization of the *hrp* cluster from *Pseudomonas syringae* pv. *syringae* 61 and Tn*phoA* tagging of genes encoding exported or membrane-spanning Hrp proteins. Mol. Plant-Microbe Interact. 4:469–476.
36. Huang, H.-C., R.-W. Lin, C.-J. Chang, A. Collmer, and W.-L. Deng. 1995. The complete *hrp* gene cluster of *Pseudomonas syringae* pv. *syringae* 61 includes two blocks of genes required for harpin$_{Pss}$ secretion that are arranged colinearly with *Yersinia ysc* homologs. Mol. Plant-Microbe Interact. 8:733–746.
37. Huang, H.-C., R. Schuurink, T. P. Denny, M. M. Atkinson, C. J. Baker, I. Yucel, S. W. Hutcheson, and A. Collmer. 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco plants. J. Bacteriol. 170:4748–4756.
38. Huguet, E., and U. Bonas. 1997. *hrpF* of *Xanthomonas campestris* pv. *vesicatoria* encodes an 87-kDa protein with homology to NolX of *Rhizobium fredii*. Mol. Plant-Microbe Interact. 10:488–498.
39. Keen, N. T. 1990. Gene-for-gene complementarity in plant-pathogen interactions. Annu. Rev. Genet. 24:447–463.
40. Keen, N. T., S. Tamaki, D. Kobayashi, D. Gerhold, M. Stayton, H. Shen, S. Gold, J. Lorang, H. Thordal-Christensen, D. Dahlbeck, and B. Staskawicz. 1990. Bacteria expressing avirulence gene D produce a specific elicitor of the soybean hypersensitive reaction. Mol. Plant-Microbe Interact. 3:112–121.
41. Kim, J. F., Z.-M. Wei, and S. V. Beer. 1997. The *hrpA* and *hrpC* operons of *Erwinia amylovora* encode components of a type III pathway that secretes harpin. J. Bacteriol. 179:1690–1697.
42. Klement, Z. 1963. Rapid detection of pathogenicity of phytopathogenic pseudomonads. Nature 199:299–300.
HR def. 43. Klement, Z. 1982. Hypersensitivity, p. 149–177. *In* M. S. Mount and G. H. Lacy (ed.), Phytopathogenic prokaryotes, vol. 2. Academic Press, Inc., New York, N.Y.
44. Kobayshi, D. Y., S. J. Tamaki, and N. T. Keen. 1989. Cloned avirulence genes from the tomato pathogen *Pseudomonas syringae* pv. *tomato* confer cultivar specificity on soybean. Proc. Natl. Acad. Sci. USA 86:157–161.
45. Laby, R. J., and S. V. Beer. 1995. Degradation of harpin by apoplastic protease activity. Phytopathology 85:1144 (Abstract).
46. Leach, J. E., and F. F. White. 1996. Bacterial avirulence genes. Annu. Rev. Phytopathol. 34:153–179.
47. Leister, R. T., F. M. Ausubel, and F. Katagiri. 1996. Molecular recognition of pathogen attack occurs inside of plant cells in plant disease resistance specified by the *Arabidopsis* genes *RPS2* and *RPM1*. Proc. Natl. Acad. Sci. USA 93:15497–15502.
48. Li, J., H. Ochman, E. A. Groisman, E. F. Boyd, F. Solomon, K. Nelson, and R. K. Selander. 1995. Relationship between evolutionary rate and cellular location among the Inv/Spa invasion proteins of *Salmonella enterica*. Proc. Natl. Acad. Sci. USA 92:7252–7256.
49. Lindgren, P. B., R. C. Peet, and N. J. Panopoulos. 1986. Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity of bean plants and hypersensitivity on nonhost plants. J. Bacteriol. 168:512–522.
50. Lonetto, M. A., K. L. Brown, K. E. Rudd, and M. J. Buttner. 1994. Analysis of the *Streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial RNA polymerase sigma factors involved in the regulation of extracytoplasmic functions. Proc. Natl. Acad. Sci. USA 91:7573–7577.
51. Lorang, J. M., and N. T. Keen. 1995. Characterization of *avrE* from *Pseudomonas syringae* pv. *tomato*: a *hrp*-linked avirulence locus consisting of at least two transcriptional units. Mol. Plant-Microbe Interact. 8:49–57.
52. Macnab, R. M. 1996. Flagella and motility, p. 123–145. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, vol. 1. ASM Press, Washington, D.C.
53. Mansfield, J., C. Jenner, R. Hockenhull, M. A. Bennett, and R. Stewart. 1994. Characterization of *avrPphE*, a gene for cultivar-specific avirulence from *Pseudomonas syringae* pv. *phaseolicola* which is physically linked to *hrpY*, a new *hrp* gene identified in the halo-blight bacterium. Mol. Plant-Microbe Interact. 7:726–739.
54. Marenda, M., F. Van Gijsegem, M. Arlat, C. Zischek, P. Barberis, J. C. Camus, P. Castello, and C. A. Boucher. 1996. Genetic and molecular dissection of the *hrp* regulon of *Ralstonia* (*Pseudomonas*) *solanacearum*, p. 165–172. *In* G. Stacey, B. Mullin, and P. M. Gresshoff (ed.), Advances in molecular genetics of plant-microbe interactions, vol. 3. APS Press, St. Paul, Minn.
55. Midland, S. L., N. T. Keen, J. J. Sims, M. M. Midland, M. M. Stayton, V. Burton, M. J. Smith, E. P. Mazzola, K. J. Graham, and J. Clardy. 1993. The structures of syringolides 1 and 2: novel C glycosidic elicitors from *Pseudomonas syringae* pv. *tomato*. J. Org. Chem. 58:2940–2945.
56. Niepold, F., D. Anderson, and D. Mills. 1985. Cloning determinants of pathogenesis from *Pseudomonas syringae* pathovar *syringae*. Proc. Natl. Acad. Sci. USA 82:406–410.
57. Oku, T., A. M. Alvarez, and C. I. Kado. 1995. Conservation of the hypersensitivity-pathogenicity regulatory gene *hrpX* of *Xanthomonas campestris* and *X. oryzae*. DNA Sequence 5:245–249.
58. Pirhonen, M. U., M. C. Lidell, D. L. Rowley, S. W. Lee, S. Jin, Y. Liang, S. Silverstone, N. T. Keen, and S. W. Hutcheson. 1996. Phenotypic expression of *Pseudomonas syringae avr* genes in *E. coli* is linked to the activities of the *hrp*-encoded secretion system. Mol. Plant-Microbe Interact. 9:252–260.
59. Plano, G. V., S. S. Barve, and S. C. Straley. 1991. LcrD, a membrane-bound regulator of the *Yersinia pestis* low-calcium response. J. Bacteriol. 173:7293–7303.
60. Preston, G., H.-C. Huang, S. Y. He, and A. Collmer. 1995. The HrpZ proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea*, and *tomato* are encoded by an operon containing *Yersinia ysc* homologs and elicit the hypersensitive response in tomato but not soybean. Mol. Plant-Microbe Interact. 8:717–732.
61. Roine, E., W. Wei, J. Yuan, E.-L. Nurmiaho-Lassila, N. Kalkkinen, M. Romantschuk, and S. Y. He. 1997. HrpA, a structural protein of a novel bacterial pilus required for plant-bacterial interaction. Proc. Natl. Acad. Sci. USA 94:3459–3464.
62. Rosqvist, R., K. E. Magnusson, and H. Wolf-Watz. 1994. Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. EMBO J. 13:964–972.
63. Russel, M. 1995. Moving through membranes with filamentous phages. Trends Microbiol. 3:223–228.
64. Salmeron, J. M., and B. J. Staskawicz. 1993. Molecular characterization and *hrp*-dependence of the avirulence gene *avrPto* from *Pseudomonas syringae* pv. tomato. Mol. Gen. Genet. 239:6–10.
65. Salmond, G. P. C., and P. J. Reeves. 1993. Membrane traffic wardens and protein secretion in gram-negative bacteria. Trends Biochem. Sci. 18:7–12.
66. Scofield, S. R., C. M. Tobias, J. P. Rathjen, J. H. Chang, D. T. Lavelle, R. W. Michelmore, and B. J. Staskawicz. 1996. Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. Science 274:2063–2065.
67. Sory, M.-P., and G. R. Cornelis. 1994. Translocation of a hybrid YopE-adenylate cyclase from *Yersinia enterocolitica* into HeLa cells. Mol. Microbiol. 14:583–594.
68. Staskawicz, B. J., F. M. Ausubel, B. J. Baker, J. G. Ellis, and J. D. G. Jones. 1995. Molecular genetics of plant disease resistance. Science 268:661–667.
69. Staskawicz, B. J., D. Dahlbeck, and N. T. Keen. 1984. Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinea* determines race specific incom-

patibility on *Glycine max* (L.) Merr. Proc. Natl. Acad. Sci. USA 81:6024–6028.
70. Tang, X., R. D. Frederick, J. Zhou, D. A. Halterman, Y. Jia, and G. B. Martin. 1996. Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. Science 274:2060–2062.
71. Turner, J. G., and A. Novacky. 1974. The quantitative relation between plant and bacterial cells involved in the hypersensitive reaction. Phytopathology 64:885–890.
72. Van den Ackerveken, G., E. Marois, and U. Bonas. 1996. Recognition of the bacterial avirulence protein AvrBs3 occurs inside the host plant cell. Cell 87:1307–1316.
73. Van Gijsegem, F., S. Genin, and C. Boucher. 1993. Evolutionary conservation of pathogenicity determinants among plant and animal pathogenic bacteria. Trends Microbiol. 1:175–180.
74. Van Gijsegem, F., C. Gough, C. Zischek, E. Niqueux, M. Arlat, S. Genin, P. Barberis, S. German, P. Castello, and C. Boucher. 1995. The *hrp* gene locus of *Pseudomonas solanacearum*, which controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex. Mol. Microbiol. 15:1095–1114.
75. Vivian, A., and J. Mansfield. 1993. A proposal for a uniform genetic nomenclature for avirulence genes in phytopathogenic pseudomonads. Mol. Plant-Microbe Interact. 6:9–10.
76. Wattiau, P., S. Woestyn, and G. R. Cornelis. 1996. Customized secretion chaperones in pathogenic bacteria. Mol. Microbiol. 20:255–262.
77. Wei, Z.-M., and S. V. Beer. 1993. HrpI of *Erwinia amylovora* functions in secretion of harpin and is a member of a new protein family. J. Bacteriol. 175:7958–7967.
78. Wei, Z.-M., and S. V. Beer. 1995. *hrpL* activates *Erwinia amylovora hrp* gene transcription and is a member of the ECF subfamily of σ factors. J. Bacteriol. 177:6201–6210.
79. Wei, Z.-M., R. J. Laby, C. H. Zumoff, D. W. Bauer, S. Y. He, A. Collmer, and S. V. Beer. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science 257:85–88.
80. Wengelnik, K., C. Marie, M. Russel, and U. Bonas. 1996. Expression and localization of HrpA1, a protein of *Xanthomonas campestris* pv. vesicatoria essential for pathogenicity and induction of the hypersensitive response. J. Bacteriol. 178:1061–1069.
81. Wengelnik, K., G. Van den Ackerveken, and U. Bonas. 1996. HrpG, a key *hrp* regulatory protein of *Xanthomonas campestris* pv. *vesicatoria*, is homologous to two-component response regulators. Mol. Plant-Microbe Interact. 9:704–712.
82. Wengelnik, K., and U. Bonas. 1996. HrpXv, and AraC-type regulator, activates expression of five of the six loci in the *hrp* cluster of *Xanthomonas campestris* pv. vesicatoria. J. Bacteriol. 178:3462–3469.
83. Whalen, M. C., R. E. Stall, and B. J. Staskawicz. 1988. Characterization of a gene from a tomato pathogen determining hypersensitive resistance in non-host species and genetic analysis of this resistance in bean. Proc. Natl. Acad. Sci. USA 85:6743–6747.
84. Wood, M. W., R. Rosqvist, P. B. Mullan, M. H. Edwards, and E. E. Galyov. 1996. SopE, a secreted protein of *Salmonella dublin*, is translocated into the target eukaryotic cell via a *sip*-dependent mechanism and promotes bacterial entry. Mol. Microbiol. 22:327–338.
85. Xiao, Y., S. Hue, J. Yi, Y. Lu, and S. W. Hutcheson. 1994. Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. syringae Pss61 *hrp* and *hrmA* genes. J. Bacteriol. 176:1025–1036.
86. Yang, Y., and D. W. Gabriel. 1995. *Xanthomonas* avirulence/pathogenicity gene family encodes functional plant nuclear targeting signals. Mol. Plant-Microbe Interact. 8:627–631.
87. Young, S. A., F. F. White, C. M. Hopkins, and J. E. Leach. 1994. AvrXa10 protein is in the cytoplasm of *Xanthomonas oryzae* pv. *oryzae*. Mol. Plant-Microbe Interact. 7:799–804.
88. Yuan, J., and S. Y. He. 1996. The *Pseudomonas syringae* Hrp regulation and secretion system controls the production and secretion of multiple extracellular proteins. J. Bacteriol. 178:6399–6402.
89. Zupan, J. R., and P. Zambryski. 1995. Transfer of T-DNA from *Agrobacterium* to the plant cell. Plant Physiol. 107:1041–1047.

Isolation of the *hreX* Gene Encoding the HR Elicitor Harpin (Xcp) from *Xanthamonas campestris* pv. *pelargonii*. S. SWANSON and Z-M. Wei. EDEN Bioscience Corporation, Bothell, WA 98011 USA.
Phytopathology 90:S75. Publication no. P-2000-0537-AMA.

This study reports the isolation of a gene encoding a proteinaceous HR elicitor from *Xanthomonas campestris* pv. *pelargonii, Xcp*. The HR elicitor exhibits a high potency for eliciting HR in tobacco. Treatment of the *Xcp* HR Elicitor with proteases resulted in a loss of HR activity. Degenerate oligonucleotides were designed based on amino acid sequences obtained from the purified HR elicitor and used to screen a *Xanthomonas campestris* pv. *pelargonii* genomic library. An open reading frame, ORF, was identified consisting of 381 base pairs that encoded a protein of 126 amino acids. The ORF initiated with a typical methionine start codon and was preceded by a putative ribosome-binding site. The ORF was designated as the *hreX* gene, encoding the HR elicitor harpin (Xcp). HreX has a molecular weight of 13.3KD, a theoretical pI of 3.8 and is glycine rich. Further studies of harpin (Xcp) and its bioactivity are currently underway.

Isolation of the *hreX* Gene Encoding the HR Elicitor Harpin$_{Xcp}$ from *Xanthamonas campestris* pv. *pelargonii*.

S. SWANSON, Z-M. Wei.
EDEN Bioscience Corporation, Bothell, WA 98011 USA

EDEN BIOSCIENCE

Introduction

The last decade has been witness to the identification, isolation and characterization of a number of hypersensitive response elicitors from plant pathogens (HR elicitors). HR elicitors have been reported from species of *Erwinia*, *Pseudomonas*, and *Ralstonia*. For the most part, the HR elicitors reported have been classified as belonging to the harpin protein family. Harpin proteins elicit the hypersensitive response in tobacco and other non-host plants, and are glycine rich, and heat-stable. They are secreted via a type III secretion system. Harpin proteins are generally located in or approximate to an *hrp* gene cluster, a highly conserved region of genes that encode regulatory factors and other components of the type III secretion system. In most of the species above, identification of the *hrp* clusters preceded the identification of the harpin proteins. The *hrp* gene clusters from several species of *Xanthomonas* have also been identified and highly studied, though to date an HR elicitor from *Xanthomonas* has not been reported. The current study reports the isolation of a gene encoding an HR elicitor from *Xanthomonas campestris pv. pelargonii* (*Xcp*).

Proteinaceous HR Elicitor

Figure 1.
Wild type *Xcp* was grown in a minimal media that was optimized to induce the expression of the HR elicitor. The induced culture was centrifuged, and the cell pellet resuspended and sonicated. The sonicate was then heat-treated, cooled and centrifuged. The resulting supernatant, or cell-free-elicitor-preparation (CFEP), was infiltrated into tobacco leaves and a hypersensitive response was observed. When samples of the CFEP were incubated in the presence of a protease prior to infiltration, no symptoms of HR were observed in the tobacco plant.

Identification and Isolation of HR Elicitor

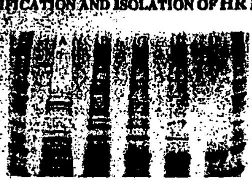


Figure 2.
CFEP was used as the starting material for the chromatographic purification of the HR elicitor. Purification consisted of two hydrophobic interaction steps; a cation exchange step, and a reverse phase chromatography polishing step (RPC). The final RPC step yielded a nearly homogeneous *Xcp* HR elicitor.
Lane A, CFEP loaded on to Butyl Seph.; lane B, HR active fraction from the Butyl Seph. loaded onto Mono S; lane C, HR active fraction from Mono S loaded on to Phenyl Seph.; lane D, HR active fraction from Phenyl Seph. loaded on to an RPC column; lane E, HR active fraction from the final RPC step, representing the purified *Xcp* HR elicitor.

Gene Isolation

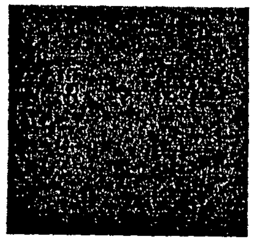


Figure 3.
The purified HR elicitor was proteolytically digested and the resulting peptides separated. Amino acid sequencing of one of the peptides yielded an unambiguous sequence of nine amino acids. A fully degenerate pool of oligonucleotides was made based on the amino acid sequence. The oligonucleotide pool was 5' end radioisotope labeled and used as a probe against an *Xcp* genomic library.
The *Xcp* genomic library was constructed in pBluescript II that contained 9 to 12 kb *Xcp* Sau3 A1 genomic DNA fragments. The library was plated and the DNA transferred to nylon membranes and fixed. The membranes were hybridized with the previously described probe. Approximately 7000 colonies were screened, and 30 colonies were isolated for further analysis. Examples of positive hybridizing DNA from library constructs are circled.

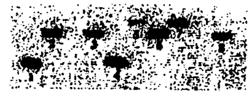


Figure 4.
Plasmids were isolated from colonies that showed positive hybridization with the HR elicitor probe. A Southern analysis was performed on the digested plasmids in order to confirm the veracity of the colony hybridization (Figure 5). Samples 2, 4, and 6 were further analyzed.

Figure 5.
Genomic DNA inserts from samples 2, 4, and 6 (Figure 4) were digested and subcloned. The subclones were constructed in pBluescript II and contained 1.5 to 3 kb genomic inserts. A final Southern analysis was performed on the subclones. This confirmed the presence of the genomic DNA fragments which hybridized with the HR elicitor probe.

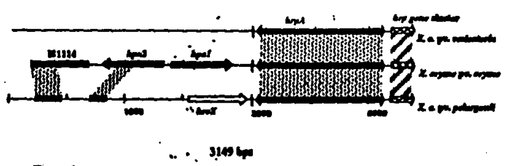


Figure 6.
DNA sequencing was performed on the inserts of the final constructs which hybridized with the HR elicitor probe (Figure 5; samples 2a, 4a, and 6a). A 378 base pair open-reading-frame (ORF) encoded a 126 amino acid protein was identified. The deduced protein had a molecular weight of ca. 12 kDa, a theoretical pI of 3.5, and was glycine rich. These characteristics match those of the originally purified *Xcp* HR elicitor protein. The ORF was designated *hreX*. The *hreX* gene product, HreX, consisted of 15.8% glycine, 18.4% glutamine, 11.4% serine, and one cysteine residue (0.9%). Sequencing of the regions flanking of *hreX* revealed that *hreX* was located immediately downstream of a *hrpA* homologue. Based on *hrp* gene clusters reported for other *Xanthomonas* species, it would seem likely that *hreX* is located just outside of the *hrp* gene cluster. BLAST analysis did not identify any genes with significant sequence similarities to the *hreX* gene. Significant homologies were observed between regions upstream of the *hreX* gene and IS1114 and *hpa2* from *X. oryzae pv. oryzae* (1).

hreX Homologue

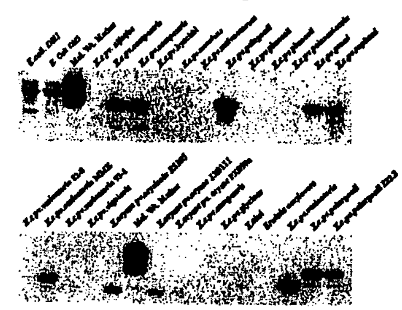


Figure 7.
Various species of *Xanthomonas* and other bacteria were obtained and screened for the presence of an *hreX* homologue. Southern analyses were performed with *hreX* probe. Hybridization with the *hreX* probe was observed in the genomic DNA of the bacterial species appearing in bold type.

Bioactivity of HreX

HreX was expressed in *E. coli*, and prepared in an identical fashion to the previously described CFEP, to generate a crude HreX extract. When infiltrated into tobacco and several other plant species, the HreX extract induced the hypersensitive response. Experiments have also been conducted with crude HreX extract to evaluate its ability to induce resistance to tobacco mosaic virus and to induce plant growth enhancement in tomato. Three days after the treatment of tobacco plants with the crude HreX extract, tobacco plants were inoculated with tobacco mosaic virus (TMV). Four days after TMV inoculation, the treated plants were observed to have 75% fewer TMV-caused lesions compared to the buffer treated control plants. Tomato plants grown from seed soaked in the crude HreX extract were observed to have an average growth 15-20% greater than the buffer treated control plants (determined by measuring plant height).

Figure 8.
Additional experiments were conducted with HreX to evaluate its ability to induce disease resistance in geraniums. Geraniums (variety Rose Star) were treated with varying concentrations of a crude HreX extract (HreX #1, 2, & 3). Five days after treatment, the geranium plants were inoculated with *Xanthomonas campestris pv. pelargonii* (100 ml at 1x10^6 cfu per ml). Fifteen days after inoculation, the HreX solution was determined to be effective in controlling the disease.

Conclusions

- A hypersensitive response elicitor was purified to near homogeneity from *Xanthomonas campestris pv. pelargonii*.
- The gene corresponding to the *Xcp* HR elicitor was isolated and designated *hreX*.
- The *hreX* gene is located next to *hrpA* just outside of the hrp gene cluster.
- BLAST analysis did not identify any genes with significant sequence similarities to the *hreX* gene. Significant homologies were observed between regions upstream of the *hreX* gene and IS1114 and *hpa2* from *X. oryzae pv. oryzae* (1).
- Sequences similar to *hreX* have been identified by Southern analysis in various other species of *Xanthomonas* including some pathovars of *X. c. pv. vesicatoria* and *X. oryzae*.
- HreX was expressed in *E. coli* and induced a hypersensitive response when infiltrated into tobacco.
- Treatment of tobacco and geranium plants with a crude HreX extract resulted in increased disease resistance. Treatment of tomato plant with the HreX extract resulted in plant growth enhancement.
- Studies are currently underway to determine whether HreX is secreted via the type III secretion system.

1. Zhu, W., Magbanua, M. M., and F. F. White. 2000. Identification of Two Novel *hrp*-Associated Genes in *hrp* Gene Cluster of *Xanthomonas oryzae* pv. *oryzae*. J. Bacteriol. 179:5655-5662.

Molecular Microbiology (1996) 20(3), 681–683

MicroCorrespondence

Unified nomenclature for broadly conserved *hrp* genes of phytopathogenic bacteria

Sir,

Genes of plant-pathogenic bacteria controlling hypersensitive response (HR) elicitation and pathogenesis were designated '*hrp*' by Lindgren *et al.* in 1986 (*J Bacteriol* 168: 512–522). *hrp* genes have been characterized in several species of the four major genera of Gram-negative plant pathogens, *Erwinia*, *Pseudomonas*, *Ralstonia* (a new proposed genus including *Pseudomonas solanacearum*) and *Xanthomonas*. To date, *hrp* genes have been found mainly in large clusters, and they have been shown to be conserved physically and, in many cases, functionally among different bacteria. Hybridization studies and genetic analyses have revealed the presence of functional *hrp* genes even in species that are not typically observed to elicit an HR, such as *Erwinia chrysanthemi* and *Erwinia stewartii*, suggesting that *hrp* genes may be common to all Gram-negative plant pathogens, possibly excluding *Agrobacterium* spp. Current knowledge of *hrp* genes has been reviewed by Bonas (1994, *Curr Top Microbiol Immunol* 192: 79–98) and by Van Gijsegem *et al.* (1995, In *Pathogenesis and Host–Parasite Specificity in Plant Diseases: Histopathological, Biochemical, Genetic and Molecular Basis.* Volume 1. (Kohmoto *et al.*, eds); Oxford: Pergamon Press, pp. 273–292).

The nucleotide sequences of four *hrp* gene clusters, those of *Ralstonia solanacearum* (previously *P. solanacearum*) (Genin *et al.*, 1992, *Mol Microbiol* 6: 3065–3076; Gough *et al.*, 1992, *Mol Plant–Microbe Interact* 5: 384–389; Gough *et al.*, 1993, *Mol Gen Genet* 239: 378–392; Van Gijsegem *et al.*, 1995, *Mol Microbiol* 15: 1095–1114), *Erwinia amylovora* (Bogdanove *et al.*, 1996, *J Bacteriol* 178: 1720–1730; Wei and Beer, 1993, *J Bacteriol* 175: 7958–7967; Wei and Beer, 1995, *J Bacteriol* 177: 6201–6210; Wei *et al.*, 1992, *Science* 257: 85–88; S. V. Beer, unpublished), *Pseudomonas syringae* pv. *syringae* (Huang *et al.*, 1992, *J Bacteriol* 174: 6878–6885; Huang *et al.*, 1993, *Mol Plant–Microbe Interact* 6: 515–520; Huang *et al.*, 1995, *Mol Plant–Microbe Interact* 8: 733–746; Lidell and Hutcheson, 1994, *Mol Plant–Microbe Interact* 7: 488–497; Preston *et al.*, 1995, *Mol Plant–Microbe Interact* 8: 717–732; Xiao *et al.*, 1994, *J Bacteriol* 176: 1025–1036), and *Xanthomonas campestris* pv. *vesicatoria* (Fenselau *et al.*, 1992, *Mol Plant–Microbe Interact* 5: 390–396; Fenselau and Bonas, 1995, *Mol Plant–Microbe Interact* 8: 845–854; U. Bonas, unpublished), have been largely determined. These clusters each contain more than twenty genes, many of which encode components of a novel protein-secretion pathway designated 'type III'. It has been shown directly that various extracellular proteins involved in pathogenesis and defence elicitation by plant-pathogenic bacteria utilize this pathway (Arlat *et al.*, 1994, *EMBO J* 13: 543–553; He *et al.*, 1993, *Cell* 73: 1255–1266; Wei and Beer, 1993, ibid.), and the pathway is known to function in the export of virulence factors from the animal pathogens *Salmonella typhimurium*, *Shigella flexneri*, and *Yersinia enterocolitica*, *Yersinia pestis*, and *Yersinia pseudotuberculosis* (for reviews, see Salmond and Reeves, 1993, *Trends Biochem Sci* 18: 7–12; and Van Gijsegem *et al.*, 1993, *Trends Microbiol* 1: 175–180). Nine type III secretion genes are conserved among all four of the plant pathogens listed above and among the animal pathogens. Based on sequence analysis and some experimental evidence, they are believed to encode one outer-membrane protein, one outer-membrane-associated lipoprotein, five inner-membrane proteins, and two cytoplasmic proteins, one of which is a putative ATPase. All of the predicted gene products, except the outer-membrane protein, show significant similarity to components of the flagellar biogenesis complex (for reviews see Blair, 1995, *Annu Rev Microbiol* 49: 489–522; and Bischoff and Ordal, 1992, *Mol Microbiol* 6: 23–28). We herein refer to the *hrp*-encoded type III pathway as the 'Hrp pathway'.

Because *hrp* genes have been characterized independently in diverse plant-pathogenic bacteria, *hrp* gene nomenclature differs in different species, and it is not always consistent even within the same organism. Different designations are used for homologous genes, and, even worse, the same designation is used for different genes in different organisms. For example, *hrpI* of *E. amylovora* is homologous with *hrpC2* of *X. campestris* pv. *vesicatoria* and *hrpO* of *R. solanacearum*, and the homologue in *P. syringae* pv. *syringae* appears in the literature both as *hrpI* and as *hrpJ2*. Also, '*hrpN*' in *R. solanacearum* designates a secretion-pathway gene, whereas in *E. amylovora*, '*hrpN*' designates the gene encoding the elicitor harpin. Furthermore, in many bacteria the number of known *hrp* genes approaches 26. In anticipation of exhausting the alphabet, some authors chose to designate *hrp* genes with a letter and a number, creating the potential for confusion of distinct genes with alleles of the same gene. For *hrp* gene researchers, the current nomenclature is at best inconvenient; for other scientists, it is bewildering.

Another problem exists: accumulation of knowledge about the structure of *hrp* loci has outpaced the accumu-

lation of information regarding the specific functions of individual genes. Typically, *hrp* loci have been identified by polar, transposon mutagenesis. Conceivably, a particular gene within an operon required for the Hrp phenotype may not be a strict Hrp determinant, but may play a more subtle role. Moreover, even phenotypes of mutations in well-characterized *hrp* genes are not the same in all pathogens. For example, although the *hrpN* gene of *E. amylovora* is required for pathogenesis of pear fruit, the homologous gene in *E. stewartii* (D. L. Coplin, unpublished) is dispensible for pathogenicity of corn. In the macerogenic bacterium *E. chrysanthemi*, even polar mutations that disrupt *hrp* secretion altogether only reduce the apparent frequency of successful infection initiation (Bauer *et al.*, 1994, *Mol Plant-Microbe Interact* 7: 573–581). Thus, the designation '*hrp*' in its strict sense, i.e., meaning required for the HR and pathogenicity, is not uniformly applicable.

At the 7th International Congress on Molecular Plant–Microbe Interactions held in Edinburgh, Scotland in 1994, a committee of *hrp* researchers and others was formed to address these problems. We, the committee members, agreed upon a system to standardize names for the subset of *hrp* genes that are broadly conserved, and agreed to broaden the definition of the '*hrp*' designation, as follows.

For the subset of *hrp* genes that are broadly conserved, the new, unique, lower-case symbol '*hrc*' will be used. The '*hr*' of *hrp* has been retained in order to evoke that name, and the '*c*' has been added to denote 'conserved.' The upper-case designations will correspond to those of the type III secretion genes of *Yersinia* spp. (for a review, see Forsberg *et al.*, 1994, *Trends in Microbiol* 2: 14–19), *yscC*, *yscJ*, *yscN*, *yscQ–U*, and *lcrD*, except that the *lcrD* homologues will be designated '*hrcV*' to avoid confusion of these as homologues of *yscD*, which is another, less well-conserved type III gene of *Yersinia* spp. We request that *Yersinia* researchers omit the letter '*V*' in naming any new *ysc* genes that might be discovered. The *ysc* nomenclature was chosen as a standard for revising *hrp* gene names for its convenient uniformity, and because, of all the genes that comprize the several known type III systems, the *Yersinia* genes show the highest degree of sequence similarity to the type III (*hrp*) genes of plant pathogens. The new names for the nine genes are given in Table 1, along with the current names in *R. solanacearum*, *E. amylovora*, *P. syringae* pv. *syringae*, and *X. campestris* pv. *vesicatoria*, and the names of homologues involved in flagellar biogenesis.

In designating genes as '*hrc*', 'broadly conserved' genes were defined as being present among the *hrp* genes of at least one representative species of each of the four plant-pathogenic genera discussed here and among the type III genes of each of the animal-pathogenic species *S. typhimurium*, *S. flexneri*, and the three yersiniae. Gene families were defined based on pairwise sequence alignments. Any two genes were considered homologous if a BESTFIT alignment (Devereux *et al.*, 1984, *Nucl Acids Res* 12: 387–395) of the predicted amino acid sequences using default parameters yielded a quality score at least five times the standard deviation above the mean quality score of 100 alignments, for each of which one of the sequences had been randomized prior to alignment (Doolittle, 1986, *Of URFs and ORFs: a Primer on How to Analyse Derived Amino Acid Sequences*. Mill Valley, California: University Science Books).

Genes that did not meet the criterion for the '*hrc*' designation will remain '*hrp*'. We have chosen to use this criterion until more data regarding structure and precise function of the products of the *hrp* and other type III genes becomes available. Some of the genes that did not meet the criterion in fact may be common to *Ralstonia*, *Erwinia*, *Pseudomonas*, and *Xanthomonas*, and have homologues in the animal pathogens, yet may be sufficiently diverged to obscure obvious homology by direct sequence comparison. As structural and functional data accrue, such relationships may become clear, and the list of *hrc* genes

Table 1. Current names and new, unified names for the broadly conserved *hrp* genes of *R. solanacearum*, *E. amylovora*, *P. syringae* pv. *syringae*, and *X. campestris* pv. *vesicatoria*. Homologues that function in flagellar biogenesis are given in the bottom row.

Unified	*hrcC*	*hrcJ*	*hrcN*	*hrcQ*	*hrcR*	*hrcS*	*hrcT*	*hrcU*	*hrcV*
R. solanacearum[a]	*hrpA*	*hrpI*	*hrpE*	*hrpQ*	*hrpT*	*hrpU*	*hrpC*	*hrpN*	*hrpO*
E. amylovora[b]	*hrcC*	*hrcJ*	*hrcN*	*hrcQ*	*hrcR*	*hrcS*	*hrcT*	*hrcU*	(*hrpI*) *hrcV*
P. syringae[c]	*hrpH*	*hrpC*	*hrpJ4*	*hrpU2/U*	*hrpW*	*hrpO*	*hrpX*	*hrpY*	(*hrpJ2*) *hrpI*
X. campestris[d]	*hrpA1*	*hrpB3*	*hrpB6*	*hrpD1*	*hrpD2*	*hrpD3*	*hrpB8*	*hrpC1*	*hrpC2*
(Flagellar)[e]		*fliF*	*fliI*	*fliY,N*	*fliP*	*fliQ*	*fliR*	*flhB*	*flhA*

a. Gough *et al.*, 1992, ibid.; Gough *et al.*, 1993, ibid.; Van Gijsegem *et al.*, 1995, ibid.
b. Bogdanove *et al.*, 1996, ibid.; Wei and Beer, 1993, ibid.; S. V. Beer, unpublished.
c. Huang *et al.*, 1992, ibid.; Huang *et al.*, *Mol Plant-Microbe Interact* 6: 515–520, 1993; Huang *et al.*, 1995, ibid.; Lidell and Hutcheson, *Mol Plant-Microbe Interact* 7: 488–497, 1994; Preston *et al.*, 1995, ibid. The predicted product of *hrpU2* aligns with the N-terminal two-thirds of a multiple alignment of the other plant- and animal-pathogen homologues; that of *hrpU* aligns with the remaining N-terminal one-third. Respectively, these genes will be designated '$hrcQ_A$' and '$hrcQ_B$.'
d. Fenselau *et al.*, 1992, ibid.; Fenselau and Bonas, 1995, ibid.; U. Bonas, unpublished. Hwang *et al.* (1992, *J Bacteriol* 174: 1923–1931) published the sequence of two genes from *Xanthomonas campestris* pv. *glycines*, designated 'ORF1' and 'ORF2,' that are homologous to hrpD1 and hrpD2 of *X. campestris* pv. *vesicatoria*, respectively.
e. For reviews, see Blair (1995, ibid.) and Bischoff and Ordal (1992, ibid.).

may grow. For any new *hrp* genes that may be discovered, we recommend the strict, sequence-alignment-based criterion for use of the '*hrc*' designation until sufficient structural and functional studies can be completed.

Some *hrp* genes are conserved only within subgroups of plant pathogens. One example is the regulatory gene *hrpB* of *R. solanacearum* (Genin *et al.*, 1992, ibid.). This gene, a member of the *araC* family, is present also in pathovars of *X. campestris* (Kamdar *et al.*, 1993, *J Bacteriol* **175**: 2017–2025; Kamoun and Kado, 1990, *J Bacteriol* **172**: 5165–5172; U. Bonas, unpublished), but absent from the *hrp* gene clusters of *P. syringae* and *E. amylovora*, which contain regulatory genes that are members of the two-component regulatory-system family (Grimm *et al.*, 1995, *Mol Microbiol* **15**: 155–165; Grimm and Panopoulos, 1989, *J Bacteriol* **171**: 5031–5038; Xiao *et al.*, 1994, ibid.; S. V. Beer, unpublished). As another example, the *hrp* gene clusters of *P. syringae* and *E. amylovora* each contain a homologue of the *Yersinia* gene *yopN* (Bogdanove *et al.*, 1996, ibid.), yet no homologue of this gene has been found in *R. solanacearum* or *X. campestris*. It is noteworthy that the genetic organizations of the *hrp* gene clusters of *X. campestris* and *R. solanacearum* are quite similar to, yet distinct from, those of *P. syringae* and *E. amylovora*, which resemble one another. We will not attempt a nomenclatural revision here for any of the non-*hrc* genes, but we encourage authors, wherever possible, to standardize names for such genes, at least within these subgroups, by using conventional rules for bacterial genetic nomenclature, including priority of publication, as a basis for naming homologues (Demerec *et al.*, 1966, *Genetics* **54**: 61–76). Although the same name might be used for different genes across subgroups, standardized names and the similar genetic organizations within the subgroups should greatly facilitate comparative studies and application of information learned in one species to the study of another.

As for the definition of the '*hrp*' designation, it now may include not only genes with a Hrp phenotype, but any gene associated with the Hrp pathway by function, homology, or location within a gene cluster or operon that is essential for the Hrp phenotype. We view use of the '*hrp*' designation in this larger sense as elective rather than mandatory. For example, the designation '*hpa*' has been used for Hrp-associated genes shown not to have a strict Hrp phenotype in *R. solanacearum* (Gough *et al.*, 1993, ibid.). In order to minimize confusion in the literature, we propose that this designation be maintained for such genes in this organism and in *X. campestris*. However, for *P. syringae* and the erwinias, in which gene phenotypes may differ from species to species, we propose a unified nomenclature based on the more inclusive definition of *hrp* genes presented here. We hope that this broadened definition will help us to gain a focussed understanding of the key elements underlying the varied and intricate interactions of bacteria with plants.

For convenience, and because '*hrc*' represents a subset of *hrp* genes, *hrc* and *hrp* genes collectively will be referred to in general discussion as '*hrp*', as in the phrase 'the *hrp* genes of phytopathogenic bacteria.' The combined designation '*hrp/c*' may be used to specify a small group of genes, e.g. 'The genes are arranged co-linearly with their *hrp/c* homologues in *Xanthomonas campestris* pv. *vesicatoria*.' Operons containing *hrc* genes still may be referred to as '*hrp*' operons. When discussing homologues with the same name (*hrp* or *hrc*) from more than one plant pathogen, distinctions can be made where necessary using abbreviations for the names of the different bacteria subscripted to the gene name.

The unified nomenclature for conserved *hrp* genes will benefit research in several ways. It makes the known homologies among plant pathogens explicit. It provides for easy cross-reference to other systems, particulary that of *Yersinia* spp. It facilitates writing and speaking cogently about *hrp* genes. Finally, it transforms a previously confusing jumble of gene names into a well-ordered catalogue, which is an accessible reference not only for *hrp* researchers, but also for those studying other type III secretion systems.

Adam J. Bogdanove,[1] Steven V. Beer,[1] Ulla Bonas,[2] Christian A. Boucher,[3] Alan Collmer,[1] David L. Coplin,[4] Guy R. Cornelis,[5] Hsiou-Chen Huang,[6] Steven W. Hutcheson,[7] Nickolas J. Panopoulos[8] and Frédérique Van Gijsegem[3*]

[1]*Department of Plant Pathology, 334 Plant Science, Cornell University, Ithaca, New York 14853, USA.*
[2]*CNRS Institut des Sciences Végétales, Avenue de la Terrasse, Bâtiment 23 911 98, Gif sur Yvette Cedex, France.*
[3]*INRA-CNRS Laboratoire de Biologie Moléculaire de Relations Plantes-Microorganismes, BP27, Chemin de Borde Rouge, Castanet-Tolosan Cedex F-31326, France.*
[4]*Department of Plant Pathology, The Ohio State University, Columbus, Ohio 43210-1087, USA.*
[5]*Microbial Pathogenesis Unit, International Institute of Cellular and Molecular Pathology and University of Louvain Medical Faculty, B-1200 Brussels, Belgium.*
[6]*Agricultural Biotechnology Laboratories, National Chung-Hsing University, Taichung, Taiwan 40227, Taiwan.*
[7]*Department of Plant Biology, University of Maryland, College Park, Maryland 20742, USA.*
[8]*Institute of Molecular Biology and Biotechnology, F.O.R.T.H. and Department of Biology, University of Crete, PO Box 1527, Heraklion 71110, Crete, Greece.*
**For correspondence: Tel. 61 28 50 45; Fax 61 28 50 61.*
Received 14 February, 1996; revised 26 February, 1996; accepted 28 February, 1996.

MPMI Vol. 13, No. 11, 2000, pp. 1251–1262. Publication no. M-2000-0825-01R. © 2000 The American Phytopathological Society

Regulation of *hrp* Genes and Type III Protein Secretion in *Erwinia amylovora* by HrpX/HrpY, a Novel Two-Component System, and HrpS

Zhongmin Wei, Jihyun F. Kim, and Steven V. Beer

Department of Plant Pathology, Cornell University, Ithaca, NY 14853, U.S.A.

Accepted 12 June 2000.

Two novel regulatory components, *hrpX* and *hrpY*, of the *hrp* system of *Erwinia amylovora* were identified. The *hrpXY* operon is expressed in rich media, but its transcription is increased threefold by low pH, nutrient, and temperature levels—conditions that mimic the plant apoplast. *hrpXY* is autoregulated and directs the expression of *hrpL*; *hrpL*, in turn, activates transcription of other loci in the *hrp* gene cluster (Z.-M. Wei and S. V. Beer, J. Bacteriol. 177:6201-6210, 1995). The deduced amino -acid sequences of *hrpX* and *hrpY* are similar to bacterial two-component regulators including VsrA/VsrD of *Pseudomonas* (*Ralstonia*) *solanacearum*, DegS/DegU of *Bacillus subtilis*, and UhpB/UhpA and NarX/NarP, NarL of *Escherichia coli*. The N-terminal signal-input domain of HrpX contains PAS domain repeats. *hrpS*, located downstream of *hrpXY*, encodes a protein with homology to WtsA (HrpS) of *Erwinia* (*Pantoea*) *stewartii*, HrpR and HrpS of *Pseudomonas syringae*, and other σ^{54}-dependent, enhancer-binding proteins. Transcription of *hrpS* also is induced under conditions that mimic the plant apoplast. However, *hrpS* is not autoregulated, and its expression is not affected by *hrpXY*. When *hrpS* or *hrpL* were provided on multicopy plasmids, both *hrpX* and *hrpY* mutants recovered the ability to elicit the hypersensitive reaction in tobacco. This confirms that *hrpS* and *hrpL* are not epistatic to *hrpXY*. A model of the regulatory cascades leading to the induction of the *E. amylovora* type III system is proposed.

Additional keywords: fire blight, pathogenicity, virulence.

Erwinia amylovora is the causal agent of the fire blight disease of many rosaceous plants including pear and apple (van der Zwet and Beer 1999). The bacterium infects blossoms, leaves, succulent shoots, and immature fruits. Symptoms of the infected plants include water soaking and discoloration, followed by necrosis. Sometimes the disease kills whole trees or substantial portions, resulting in devastating economic loss. In nonhost plants such as tobacco and Arabidopsis, the bacterium elicits the defensive hypersensitive reaction (HR), which is characterized by rapid, localized, cell death (Goodman and Novacky 1994). For infection and HR induction, genes generally called *hrp* (hypersensitive response and pathogenicity; see Alfano and Collmer 1996 for a review) are essential.

The *hrp* gene cluster of *E. amylovora* Ea321 has been cloned in several cosmids and enables nonpathogenic bacteria such as *Escherichia coli* to elicit the HR in plants (Beer et al. 1991). According to phenotypic analyses of mutants, *hrp* genes of *E. amylovora* are localized within a 25-kb region of DNA, consisting of at least eight transcriptional units (Wei and Beer 1993). Sequence analysis (Bogdanove et al. 1996; Kim et al. 1997) indicated that the majority of *hrp* genes encode proteins that are thought to be components of a specialized protein secretion apparatus called the type III pathway (Hrp pathway for plant pathogens) (Galán and Bliska 1996). Several proteins including harpins (HrpN and HrpW) and a pathogenicity/avirulence protein (DspE) have been shown to be secreted via the pathway (Bogdanove et al. 1998a; Kim and Beer 1998; Wei and Beer 1993).

Transcriptional expression of *hrp* genes is induced under conditions similar to the environment of the plant apoplast: low carbon and nitrogen, low pH (5.5), and low temperature (18°C) (Wei et al. 1992). Two independent loci, complementation groups IV and V, in the *hrp* cluster were found to have regulatory function (Sneath et al. 1990; Wei and Beer 1993, 1995). Mutations in these loci abolish harpin production and the HR-eliciting and disease-causing abilities of *E. amylovora* (Wei and Beer 1993). Preliminary sequence analysis indicated that one of them (group IV) contains a gene called *hrpS* (Sneath et al. 1990) that encodes a protein similar to σ^{54}-dependent transcriptional activators (Morett and Segovia 1993). Complementation group V encodes *hrpL* (Wei and Beer 1995), which is homologous to genes encoding members of the ECF subfamily of eubacterial sigma factors (Lonetto et al. 1994). HrpL recognizes conserved promoter sequences called "*hrp* boxes" (Xiao and Hutcheson 1994), and directs the transcription of other pathogenicity genes including *hrp* secretion operons (*hrpA*, *hrpC*, and *hrpJ*) (Wei and Beer 1995), harpin genes (*hrpN* and *hrpW*) (Kim and Beer 1998; Wei and Beer 1995), and a disease-specific locus (*dspEF* [Bogdanove et al. 1998b]; *dspAB* [Gaudriault et al. 1997]).

Corresponding author: S. V. Beer; Telephone: +1-607-255-7870; Fax: +1-607-255-4471; E-mail: svb1@cornell.edu

Current address of Zhongmin Wei: EDEN Bioscience Corp., 11816 North Creek Parkway, Bothell, WA 98011-8205, U.S.A.

J. F. Kim and Z. Wei contributed equally to the work and should be considered co-first authors.

Nucleotide and/or amino acid sequence data have been deposited in the GenBank data base under accession number AF083877.

Here we report the characterization of two new regulatory genes, designated *hrpX* and *hrpY*, and the further analysis of *hrpS*. *hrpX* and *hrpY* are present in an operon situated between *hrpS* and *hrpL*. Analysis of deduced protein sequences suggested that they constitute a two-component regulatory complex; HrpX functioning as a sensor and HrpY as the response-regulator partner of HrpX. *hrpX*, *hrpY*, and *hrpS* are components of a complex regulatory network that leads to activation of *hrpL* and eventually other genes in the *hrp* cluster of *E. amylovora*.

RESULTS

Identification and sequence analysis of the *hrpXY* locus.

Previous studies have identified several loci, including *hrpC*, *hrpA*, *hrpS*, *hrpL*, and *hrpJ*, that are essential for the Hrp phenotype (Bogdanove et al. 1996; Kim et al. 1997; Wei and Beer 1993, 1995) (Fig. 1A). Preliminary genetic analysis of pCPP430 in *Escherichia coli* suggested the presence of a new locus, between *hrpS* and *hrpL*, that also is required for the Hrp phenotype and contains novel regulatory components. We have designated this locus *hrpXY*.

A 3.4-kb *BglII*- and *ClaI*-digested fragment of pCPP430 was cloned into pBluescript KS+, resulting in pCPP1178. The sequence of the insert of pCPP1178 revealed two tightly linked open reading frames (ORFs) between *hrpL* and *hrpS* that are capable of encoding proteins of 495 and 213 amino acid residues, respectively (Fig. 1B). These ORFs were named *hrpX* and *hrpY*, respectively. Potential ribosome-binding sites, AGGAG and TGGAA, were found 5 and 7 bp upstream of the *hrpX* and *hrpY* start codons, respectively. Although the ribosome-binding site ahead of *hrpY* weakly matches the consensus sequence, we assume it is sufficient for translation of *hrpY*; only a 4-bp space exists between the *hrpX* stop codon and *hrpY* start codon and translational coupling is plausible. To confirm that the *hrpX* and *hrpY* ORFs produce proteins, pCPP1178 was placed in a gene expression system mediated by the T7 RNA polymerase. Two distinct protein bands were visible following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The apparent molecular masses of HrpX and HrpY were about 50 and 25 kDa, respectively (data not shown), close to the sizes expected from the deduced amino acid sequences.

The start codon of *hrpX* is located 146 bp downstream of the *hrpL* stop codon, and a promoter prediction program (see Materials and Methods) identified two putative σ^{70} promoter sequences, TAGACG-N_{17}-TAAAGT (score from promoter prediction by neural network = 0.97) and TTGCAA-N_{16}-CCTAAT (score = 0.95), 111 and 33 bp upstream of the *hrpX* start codon, respectively. There is a 361-bp noncoding region between *hrpY* and *hrpS*. Palindromic sequences that may serve either as targets of regulatory components or as transcription terminators, GTAAACANTGTTTAC and GGATAAAATGG-TTGTGG-N_7-CCGCTTCCATTTTATCC, were identified in the *hrpL-hrpX* and *hrpY-hrpS* intergenic regions, respectively. The tight linkage of *hrpX* and *hrpY*, and the existence of long noncoding areas and inverted repeats upstream of *hrpX* and downstream of *hrpY*, suggest that the two genes form an operon.

HrpX and HrpY constitute a two-component regulatory system.

Comparison of the predicted amino acid sequences of *hrpX* and *hrpY* with sequences in the data bases revealed significant similarities with many two-component regulatory proteins. The homologs include VsrA/VsrD of *Pseudomonas* (now *Ralstonia*) *solanacearum*, which regulate virulence gene expression (Huang et al. 1995b); UhpB/UhpA of *Escherichia coli*, which participate in the regulation of sugar transport (Friedrich and Kadner 1987); NarX/NarP,NarL of *Escherichia*

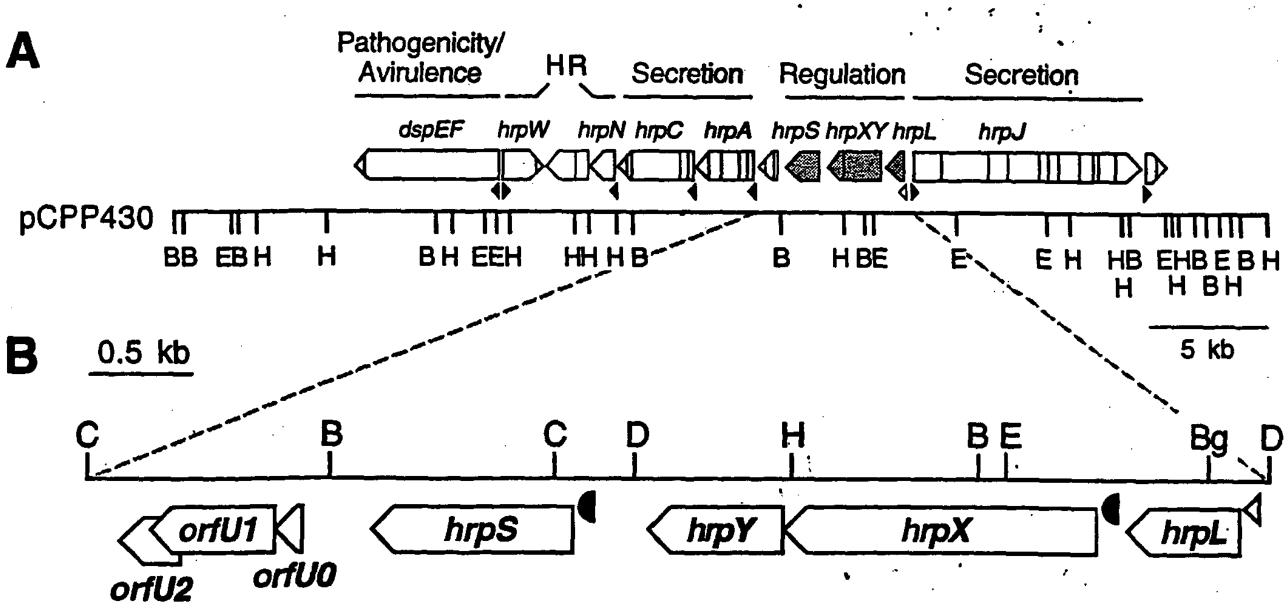


Fig. 1. A, Operon organization of the *hrp/dsp* gene cluster of *Erwinia amylovora* cloned in pCPP430. B, Central region covering regulatory genes *hrpL*, *hrpX*, *hrpY*, and *hrpS*. Boxes and arrow boxes: transcriptional units or open reading frames; names of the characterized operons or genes are given above, inside, or below. Filled triangles: putative HrpL-dependent promoters. Open triangles: putative σ^{54} promoters. Closed half circles: putative σ^{70} promoters. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Bg, *Bgl*II; C, *Cla*I; and D, *Dra*I.

coli, which are involved in the regulation of anaerobic respiratory gene expression (Rabin and Stewart 1993); and DegS/DegU of *Bacillus subtilis*, which are involved in extracellular enzyme production (Kunst et al. 1988) (Fig. 2; Table 1). In addition, HrpY showed high sequence similarity with many other transcriptional activators including ExpA of *E. carotovora* (33% identity), which is involved in global control of virulence (Eriksson et al. 1998); UvrY of *Escherichia coli* (33% identity) (Sharma et al. 1986); SirA of *Salmonella typhimurium* (32% identity) (Johnston et al. 1996); and GacA of several animal- and plant-associated *Pseudomonas* spp. (29 to 30% identities) (Laville et al. 1992).

The high sequence similarity of HrpX with histidine kinases suggests that HrpX is a sensor. HrpX has the conserved His

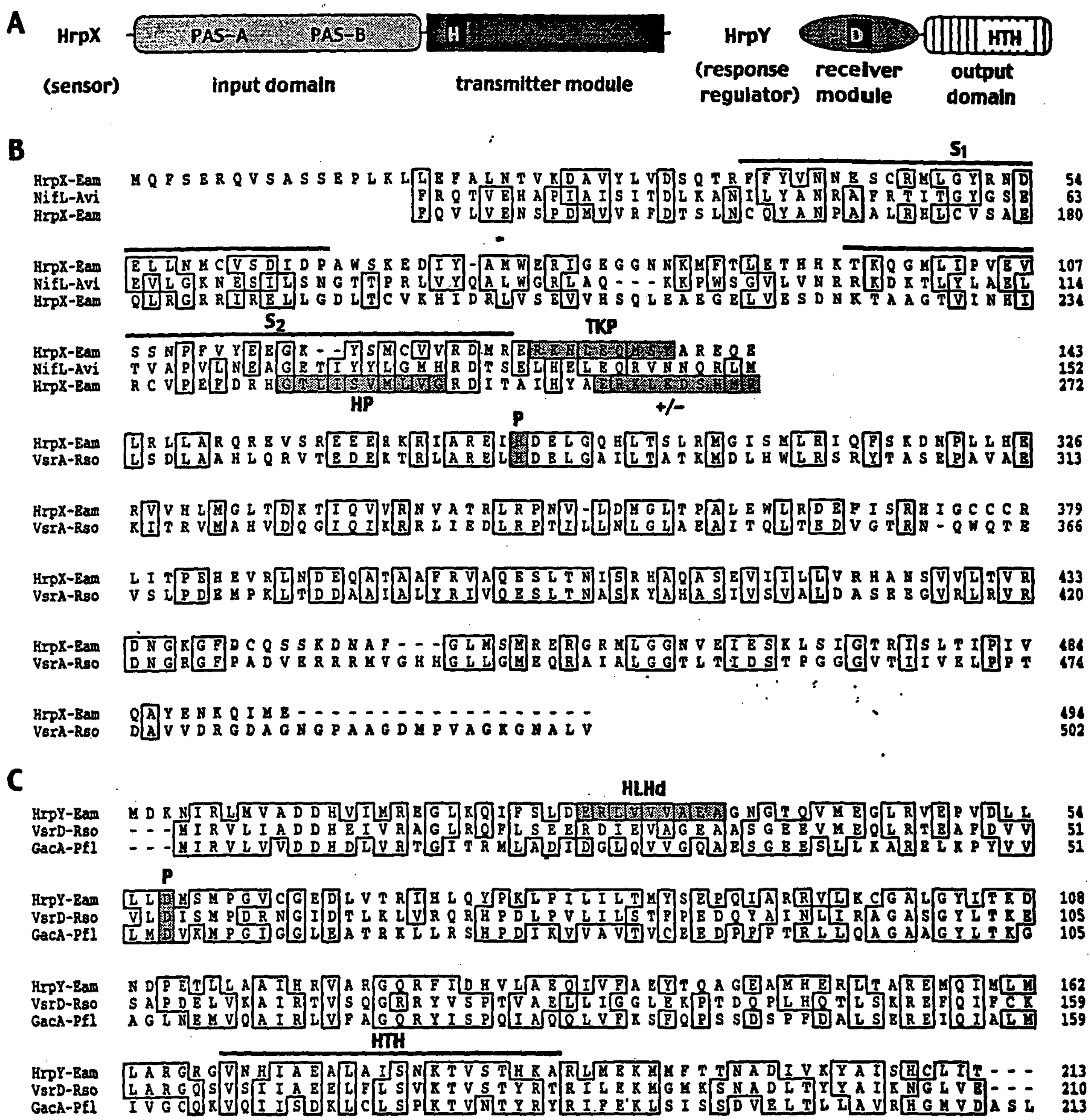


Fig. 2. A, Diagramatic illustrations of HrpX and HrpY of *Erwinia amylovora* showing predicted domain structures. **B** and **C,** Alignment of HrpX and HrpY with similar proteins. Designations of diagrams are after Parkinson and Kofoid (1992). PAS-A and PAS-B denote two repeats of the PAS domain, H and D phosphorylated histidine and aspartate residues, respectively, and HTH the helix-turn-helix DNA-binding motif. Overlines represent the S-boxes in the PAS domain (S_1 and S_2) and the HTH motif. A putative tyrosine kinase phosphorylation site (TKP), a hydrophobic region (HP), a putative charge-rich linker (+/–), and a helix-loop-helix dimerization domain signature (HLHd), and phosphorylation sites (P) are shown by shading. PILEUP program (GCG software package, vers. 7.3; Genetics Computer Group, Madison, WI, U.S.A.) with default parameters was used to align the sequences. Accession numbers of the compared proteins: NifL of *Azotobacter vinelandii*, SWISS-PROT:P30663; VsrA and VsrD of *Ralstonia solanacearum*, PIR:S41544 and PIR:I40540; and GacA of *Pseudomonas fluorescens*, SWISS-PROT:P32967. In the HrpX alignment, only 27 to 152 residues of NifL and C-terminal 243 residues of VsrA are shown. Also, the HrpX sequence is shown on two lines for the first three rows.

residue for autophosphorylation and a hydrophobic domain that may enable the protein to be transiently associated with the cytoplasmic membrane (Fig. 2B). The C-terminal putative transmitter domain (residues 273 to 494) of HrpX shows most similarity to the kinase domains of the sensor proteins listed in Table 1; the N-terminal putative input domain of HrpX shows similarity to PAS domains (Zhulin et al. 1997) of *Methanobacterium thermoautotrophicum*, *Azotobacter vinelandii*, and other organisms. Several PAS-containing proteins are sensors of bacterial two-component systems. The PAS domain typically consists of two direct sequence repeats (PAS-A and PAS-B), and each repeat contains two highly conserved regions called S_1 and S_2 boxes (Zhulin et al. 1997). In the case of HrpX, the second repeat (PAS-B) seems imperfect (Fig. 2B). Based on ScanProsite analysis (Appel et al. 1994), another feature of HrpX with unknown functional relevance is a putative tyrosine kinase phosphorylation site (PROSITE:PS00007).

HrpY appears to be a response regulator with a putative receiver domain at the N terminus (up to 102 amino acid residues) and a DNA-binding domain at the C terminus (Fig. 2A). As shown in Figure 2C, HrpY contains the conserved Asp residue, which may be phosphorylated by the sensor, and the

Table 1. HrpX and HrpY of *Erwinia amylovora* compared with two-component regulatory proteins (sensors/response regulators) of other bacteria

Bacterium	Protein	Amino acids	% Identity[a]
Erwinia amylovora	HrpX/HrpY	494/213	–
Ralstonia solanacearum	VsrA/VsrD	502/210	34/41
Escherichia coli	UhpB/UhpA	500/196	32/32
Bacillus subtilis	DegS/DegU	385/229	32/28
Escherichia coli	NarX/NarP, NarL	598/215, 216	31/33, 32

[a] % Identities from a BLASTP search of HrpX and HrpY with default parameters, except for no filtering for low complexicity regions. Only the transmitter domain of HrpX (residues 273 to 494) was used for comparisons with other sensor proteins.

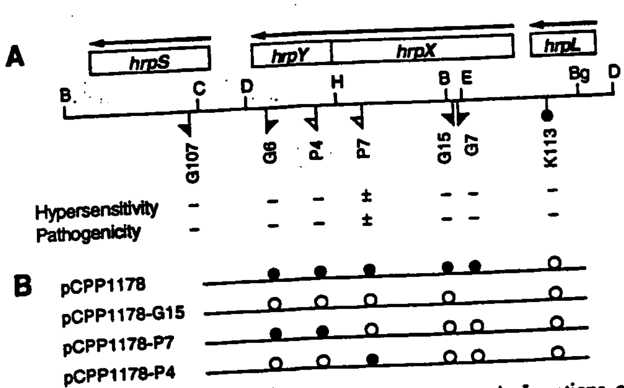


Fig. 3. Genetic characterization of the *hrpXY* locus. **A,** Locations of transposon insertions and phenotypes of *hrpX* and *hrpY* mutants of *Erwinia amylovora* Ea321. Rectangles above map of restriction enzymes and transposons represent transcriptional units. Arrows: directions of transcription. Closed flags: insertions by Tn*5-gusA1*. Open flags: insertions by Tn*phoA*. Llollipop: a Tn*10*-miniKm insertion. Mutants shown by minus signs below insertion points did not elicit the hypersensitve reaction (HR) or cause disease (Hrp⁻); a mutant shown by ± infrequently elicited spotty HR and showed low virulence. **B,** Complementation assay of *hrpX* and *hrpY* mutants of *E. amylovora* Ea321 with various plasmids. Closed circle: plasmid complemented Hrp phenotype of the mutant containing the transposon insertion in the same column. Open circle: plasmid did not change the phenotype of corresponding mutant.

helix-turn-helix DNA binding motif. HrpY also has a sequence that matches the Myc-type helix-loop-helix dimerization domain signature (PROSITE:PS00038), the functional significance of which remains to be tested.

Genetic characterization of *hrpX* and *hrpY*.

The *hrpXY* locus in pCPP430 was mutagenized with transposons Tn*5-gusA1* and Tn*phoA*. Derivatives of pCPP430 containing the transposon insertions were marker-exchanged into the genome of *E. amylovora* Ea321. All *hrpY* mutants of Ea321 failed to elicit the HR in tobacco and to infect immature pear fruits (Fig. 3A). Two classes of *hrpX* insertion mutants were obtained. Ea321-G15 and Ea321-G7, which were made with Tn*5-gusA1*, were similar to *hrpY* mutants in phenotypes. Ea321-P7, an *hrpX*::Tn*phoA* mutant, caused slight tissue collapse in tobacco at higher inoculum dose and had low virulence in immature pears, rather than the strict Hrp⁻ phenotype (Fig. 3A). Specifically, tobacco leaves infiltrated with Ea321-P7 at $\geq 5 \times 10^8$ CFU per ml developed a spotty HR 36 h after infiltration. Also, in immature pears inoculated with the mutant, bacterial ooze appeared 3 days later than in those inoculated with the wild type, and the population of the mutant recovered was only one-tenth of that of the wild type (data not shown).

Virulence of the mutants was restored to near wild-type levels by providing the mutants with pCPP1178 in *trans* (Fig. 3B). The *hrpX*::Tn*5-gusA1* mutants of Ea321 were not complemented by pCPP1178-P4 that contains a transposon insertion in *hrpY* (Fig. 3B). This suggests that *hrpX* and *hrpY* are in the same transcriptional unit and the Tn*5-gusA1* mutations in *hrpX* are polar. We found, however, that the *hrpX*::Tn*phoA* mutant Ea321-P7 can be complemented by pCPP1178-P4, indicating that the Tn*phoA* insertion of *hrpX* did not affect the function of *hrpY* (Fig. 3B). Tn*phoA*-induced mutations that permit the expression of downstream genes have been observed frequently in *E. amylovora* (Z. Wei and S. V. Beer, *unpublished data*) and *Pseudomonas syringae* (Huang et al. 1995a). Thus, we believe that the P7 insertion is nonpolar and that the peculiar phenotype of the Ea321-P7 may reflect the function of *hrpX*.

All the transposon mutations in the *hrpXY* locus were complemented by derivatives of pCPP430 with transposon insertions in *hrpS* or *hrpL* (data not shown), confirming the suggestion from sequence analysis that *hrpX* and *hrpY* constitute an independent complementation group. Based on results of sequence analysis and genetic characterization, we conclude (i) *hrpXY* is required for the Hrp phenotype, and (ii) *hrpX* and *hrpY* constitute a two-gene operon, *hrpXY*.

Expression of *hrpXY* is environmentally regulated.

A new construct, pCPP1203, was used to monitor expression of the *hrpXY* promoter in a nutrient-rich medium and a minimal medium that induces the expression of *hrp* genes (Wei et al. 1992). pCPP1203 was derived from pCPP1178-G15 (*hrpX*::Tn*5-gusA1*) in which the directions of *hrpX* and *gusA* are the same. pCPP1178-G15 was digested with *Bam*HI and *Sac*I (an *Sac*I site is present in the vector), which cuts out the *hrpXY* promoter region, a 5′ portion of the *hrpX* coding region fused to Tn*5-gusA1*, and the whole transposon. Th resulting fragment was then ligated to pCPP43, which had been digested with the same enzymes. pCPP43 (gift of David

W. Bauer) is a derivative of pOU61, which is a low-copy-number plasmid (approximately one copy per bacterium at 30°C) (Larsen et al. 1984).

In *E. amylovora* and *Escherichia coli*, the *hrpXY* promoter directed high levels of basal expression in Luria broth (LB), but expression of *hrpX*::Tn*5*-*gusA1* was enhanced threefold in the *hrp*-inducing minimal medium (IM) (Table 2). Enhanced levels of *hrpX*::Tn*5*-*gusA1* expression were also observed from assays of the strains in tobacco leaves and immature pears (data not shown). No β-glucuronidase (GUS) activity was detected for *Escherichia coli* SØ200Δ*uidA*(pCPP1203) unless functional *hrpXY* was provided (Table 2). Similarly, high basal-level expression of *hrpX*::Tn*5*-*gusA1* of Ea321(pCPP1203) in Table 2 is probably due to functional *hrpXY* present in the chromosome. The latter two observations indicate that *hrpXY* is also autoregulated.

hrpX and hrpY control the expression of hrpL.

To study the effect of *hrpX* and *hrpY* on the control of *hrpL* expression, a *hrpL*::Tn*5*-*gusA1* fusion (pCPP139-G44) (Wei and Beer 1995) was marker exchanged into an *hrpX* mutant (Ea321-P7) and an *hrpY* mutant (Ea321-P4), to generate *hrpX*-*hrpL* and *hrpY*-*hrpL* double mutants Ea321-P7G44 and Ea321-P4G44, respectively. Mutation in *hrpY* completely abolished *hrpL* expression (Fig. 4). However, the *hrpX* mutant reduced *hrpL* expression only to about 20% of its wild-type level, opening the possibility that the mutated HrpX may be still partially functional or another sensor protein may cross talk with HrpY.

Analysis of the hrpS locus and the ORFs between hrpS and hrpA.

hrpS also partially controls *hrpL* expression in *E. amylovora* and is located downstream of *hrpXY* (Wei and Beer 1995). We report here the entire nucleotide sequence of the region between *hrpY* and *hrpA*, which includes *hrpS*, to complete the preliminary results on *hrpS* presented previously (Sneath et al. 1990).

The *hrpS* locus of *E. amylovora* Ea321 contains a single-gene operon, based on the large intergenic regions beyond the coding region of *hrpS*, and a potential terminator, CGGCGACAGC-N_8-GCTGTCGCCG, that lies 49 bp downstream of the *hrpS* stop codon. The *hrpS* ORF is preceded by a potential σ^{70} promoter, GTGGCA-N_{18}-TATTAC (score from promoter prediction by neural network = 0.96), and it encodes a 324 amino acid protein. HrpS has homology to members of the σ^{54}-dependent, enhancer-binding protein family (Morett and Segovia 1993). HrpS shows highest sequence similarity with WtsA (HrpS) of *Erwinia* (*Pantoea*) *stewartii* (Frederick et al. 1993) (79% identity over 322 amino acid residues without gaps from BLASTP), HrpR and HrpS of *P. syringae* pathovars (51 to 55% identities) (Grimm et al. 1995; Xiao et al. 1994), and DctD of *Rhizobium* spp. (39% identities) (Jiang et al. 1989; Ronson et al. 1987). HrpS of *E. amylovora* has two putative ATP-binding sites at the N terminus and a helix-turn-helix DNA-binding motif at the C terminus (Fig. 5A). HrpS shows high sequence similarity to other regulators in the NtrC family throughout the entire σ^{54} interaction domain. However, similar to other HrpR/HrpS proteins, HrpS of *E. amylovora* contains a very short N-terminal A domain (Shingler 1996), and seems to lack the phosphorylation receiver domain (Fig. 5A).

In the region between *hrpS* and *hrpA*, three potential genes, designated *orfU0*, *orfU1*, and *orfU2* (Fig. 1B), were identified by application of the GeneMark.hmm algorithm (Lukashin and Borodovsky 1998). *orfU0* is a small ORF encoding a 46 amino acid basic protein, without significant similarity to any protein in the data base. Preceded by GGAGT 8 bp upstream, *orfU1* encodes a 203 amino acid basic protein that is similar to a conserved hypothetical protein HP1401 of *Helicobacter pylori* (32% identity over 164 amino acid residues with 12 gaps) (Fig. 5B). Interestingly, protein sequence of the next ORF, *orfU2*, shows even higher similarity to HP1401 (residues 189 to 229; 41% identity without gaps). This suggests the possibility that a frame shift in *orfU1*-*orfU2* resulted in the two current ORFs, and that both may be defective. The lack of an obvious promoter in front of *orfU0*, the lack of good ribosome-binding sites in front of *orfU0* and *orfU2*, the potential frame-shift mutation at the 3′ region of *orfU1*, and the lack of a phenotype of Tn*phoA*-induced *orfU1* mutants (data not shown) indicate that the region comprising *orfU0*-*orfU2* is unlikely to be functional in Ea321.

Expression of hrpS is not autoregulated, and induction of hrpS is independent of hrpX or hrpY.

An *hrpS*::*gusA1* fusion designated G107 (Wei et al. 1992) was used to assay the expression of *hrpS*. A fragment of

Table 2. Expression of the *hrpXY* promoter in Luria broth (LB) and in a *hrp*-inducing minimal medium (IM)

Bacterial strain[a]	GUS activity[b] LB	GUS activity[b] IM
Erwinia amylovora Ea321(pCPP1203)	242 ± 12	788 ± 32
E. coli SØ200Δ*uidA*(pCPP1203)	2 ± 3	3 ± 3
E. coli SØ200Δ*uidA*(pCPP1203, pCPP1178)	145 ± 19	878 ± 33

[a] *E. coli* SØ200Δ*uidA* is an *Escherichia coli* strain with no β-glucuronidase (GUS) activity due to deletion of *gusA*. pCPP1203 is a low-copy-number plasmid containing *hrpX*::Tn*5*-*gusA1*; pCPP1178 is a high-copy-number plasmid containing functional *hrpX* and *hrpY* genes.
[b] Picounits per CFU; mean of three replicates ± standard deviation.

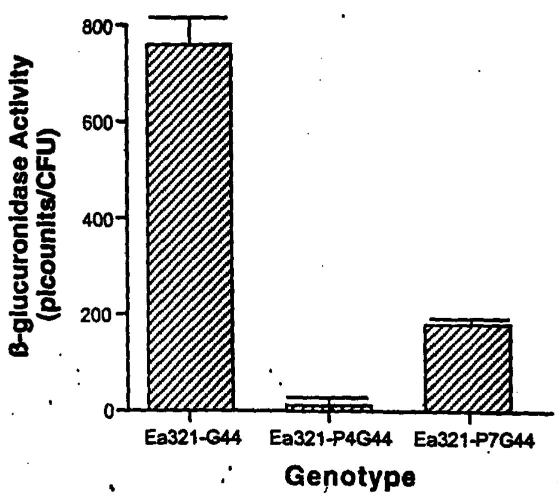


Fig. 4. Effect of mutations in *hrpX* and *hrpY* on expression of *hrpL*. Genotypes of the strains are Ea321-G44, *hrpL*::Tn*5*-*gusA1* (Wei and Beer 1995); Ea321-P4G44, *hrpY*::Tn*phoA* and *hrpL*::Tn*5*-*gusA1*; and Ea321-P7G44, *hrpX*::Tn*phoA* and *hrpL*::Tn*5*-*gusA1*. Error bars: standard deviation from three replicates. Cells grown in inducing medium (IM) were assayed (see Materials and Methods for details).

pCPP430-G107 digested with *Bam*HI contains the whole transposon, the *hrpS* gene fused to Tn*5-gusA1*, and the *hrpS* promoter region. This *Bam*HI fragment was ligated with a low-copy-number plasmid, pCPP8 (Bauer 1990), that was cut with the same enzyme. The resulting plasmid was designated pCPP1058. As with *hrpXY*, expression of *hrpS* in *Escherichia coli* or in *E. amylovora* was induced under *hrp*-inducing conditions (Table 3). However, autoregulation was not required for *hrpS* expression; the presence of functional *hrpS* did not affect the expression of a *hrpS*::*gusA1* fusion in pCPP1058 (Table 3).

To determine whether the newly discovered two-component system has any effect on the expression of *hrpS*, an *hrpS*::Tn*5-gusA1* fusion (pCPP430-G107) was marker-exchanged into *hrpX* and *hrpY* mutants. Neither *hrpX* nor *hrpY* affected *hrpS* expression significantly (Fig. 6).

hrpS and hrpL, provided by multicopy plasmids, suppress defects in hrpX or hrpY.

To further characterize the regulatory relationships between *hrpXY*, *hrpS*, and *hrpL*, the HR-impaired strains Ea321-P7, Ea321-P4, and Ea321-G107 were transformed with pCPP1178

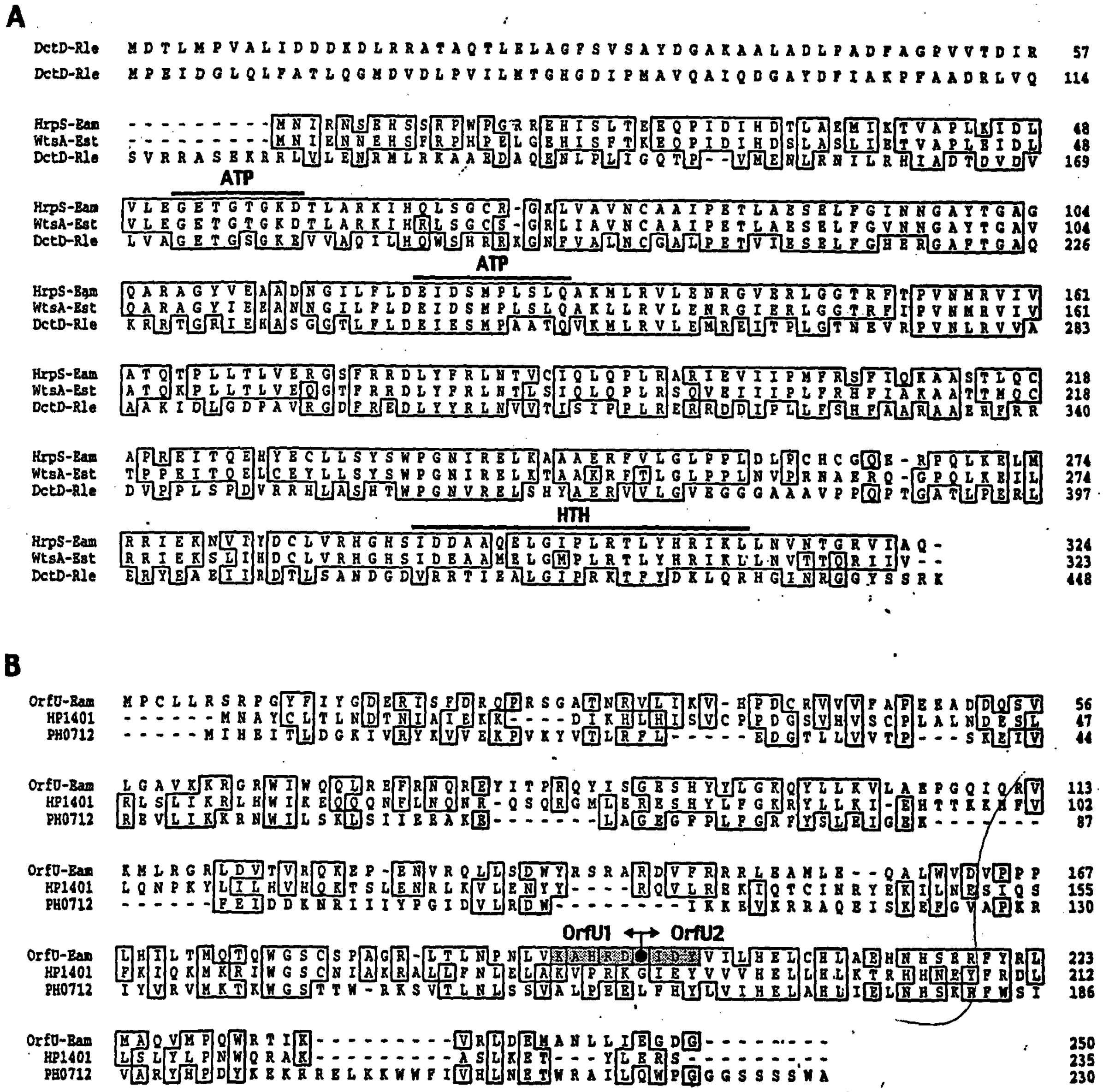


Fig. 5. Alignments of **A,** HrpS, and **B,** OrfU of *Erwinia amylovora* with similar proteins. PILEUP program (GCG software package, version 7.3; Genetics Computer Group, Madison, WI, U.S.A.) with default parameters was used to align the sequences. Overlines represent ATP-binding sites (ATP) and the helix-turn-helix DNA-binding motif (HTH). Sequence of OrfU is a composite of sequences of *orfU1* and *orfU2* products. A putative tyrosine kinase phosphorylation site (PROSITE: PS00007) is indicated by shading. Black circle in the OrfU sequence denotes location of a probable reading-frame shift. Accession numbers: WtsA of *E. stewartii*, SWISS-PROT:P36219; DctD of *Rhizobium leguminosarium*, SWISS-PROT:P10046; HP1401 of *Helicobacter pylori*, GENBANK:AE000640; and PH0712 of *Pyrococcus horikoshii*, DDBJ:AP000003.

(contains *hrpXY*), pCPP1001 (contains *hrpS*) (Wei and Beer 1995), or pCPP1078 (contains *hrpL*) (Wei and Beer 1995). The resulting transformants were infiltrated into panels of tobacco leaves to determine which, if any, of the regulatory genes, when present in multiple copies, are sufficient to restore the HR-eliciting ability to the mutants. Panels infiltrated with *hrpX* and *hrpY* mutants containing *hrpL* developed the HR (Table 4), often faster than panels infiltrated with the wild-type strain. The panels began to show collapse 8 to 12 h after infiltration; by 24 h, the whole infiltrated area had collapsed in a typical HR. This result is consistent with dependence of *hrpL* expression on *hrpX* and *hrpY*. Interestingly, similar suppression was observed from *hrpX* and *hrpY* mutants containing *hrpS*, whereas *hrpX* and *hrpY* did not restore the HR phenotype of the *hrpS* mutant (Table 4).

DISCUSSION

The HrpX/HrpY two-component protein system.

Our results demonstrate that *E. amylovora* employs the HrpX/HrpY two-component regulatory proteins to direct expression of an alternate sigma factor gene, *hrpL*, that in turn activates a type III protein secretion system. This provides for a quick change in the pattern of gene expression needed to initiate infection. HrpX is a putative I_CT-type sensor (Parkinson and Kofoid 1992) composed of the N-terminal PAS domain and the C-terminal histidine kinase domain (Fig. 2A). HrpX appears to be cytoplasmic, and may be anchored to the inner membrane by its internal hydrophobic region. Other members of the PAS-containing I_CT-type sensor kinases include NifL, NtrB, and KinA (Zhulin et al. 1997). HrpY appears to be a RO_{III} subfamily response regulator (Parkinson and Kofoid 1992). Consistent with the HrpX transmitter domain, HrpY shows significant sequence similarity to VsrD, DegU, UhpA, and NarL.

Two-component systems with PAS domains in the sensor component include NifL/NifA, DctS/DctR, and BvgS/BvgA (Zhulin et al. 1997). Among these only NifL does not contain the periplasmic domain, and HrpX is more similar to NifL than the other two. NifL and most other PAS-containing proteins are sensors (Zhulin et al. 1997), and their signal input domains are located at the N terminus (Parkinson and Kofoid 1992). Thus, HrpX may directly perceive environmental signals with its N-terminal PAS domain. One function of the PAS domain is to act as a protein dimerization motif (Kay 1997). This raises the possibility of HrpX dimerization, which is required for the functional state of two-component sensors (Parkinson and Kofoid 1992).

Two-component regulatory system and type III protein secretion.

Although the two-component system is widely used to control bacterial gene expression (Hoch and Silhavy 1995), reports of its function in regulation of the type III system are just emerging. In *S. typhimurium*, SirA is a response regulator essential for induction of *hilA*, *prgHIJK*, and *sigDE* (Hong and Miller 1998; Johnston et al. 1996), and the PhoQ/PhoP two-component system represses the expression of the *prg* locus (Pegues t al. 1995). The CpxA/CpxR system controls the pH-dependent expression of the *Shigella sonnei virF* gene, which in turn activates *ipaBCD* and *virG* (Nakayama and Watanabe

Table 3. Expression of the *hrpS* promoter in Luria broth (LB) and in *hrp*-inducing minimal medium (IM)

Bacterial strain[a]	GUS activity[b] LB	GUS activity[b] IM
E. coli SØ200Δ*uidA*(pCPP1058)	94 ± 12	367 ± 9
E. coli SØ200Δ*uidA*(pCPP1058, pCPP1001)	105 ± 17	378 ± 23
Erwinia amylovora Ea321-G107	36 ± 11	188 ± 35
Erwinia amylovora Ea321-G107(pCPP1001)	42 ± 21	229 ± 29

[a] *E. coli* SØ200Δ*uidA* is an *Escherichia coli* strain with no β-glucuronidase (GUS) activity due to deletion of *gusA*. *Erwinia amylovora* Ea321-G107 is a mutant of Ea321 containing a Tn*5-gusA1* insertion in *hrpS* (Wei et al. 1992). pCPP1058 is a low-copy-number plasmid containing *hrpX*::Tn*5-gusA1*; pCPP1001 is a high-copy-number plasmid containing the functional *hrpS* gene and its promoter (Wei and Beer 1995).

[b] Picounits per CFU; meanof three replicates ± standard deviation.

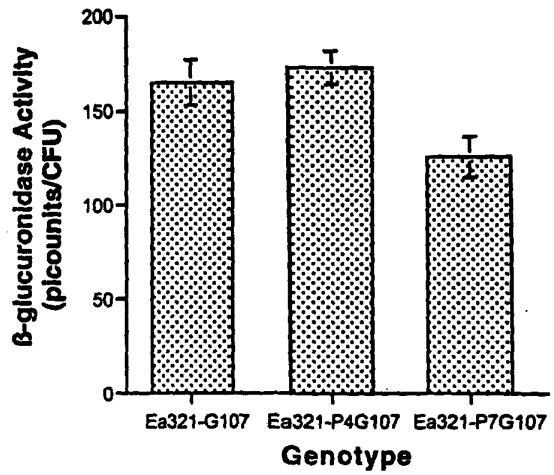


Fig. 6. Effect of mutations in *hrpX* and *hrpY* on expression of *hrpS*. Genotypes of the strains are Ea321-G107, *hrpS*::Tn*5-gusA1* (Wei et al. 1992); Ea321-P4G107, *hrpY*::Tn*phoA* and *hrpS*::Tn*5-gusA1*; and Ea321-P7G107, *hrpX*::Tn*phoA* and *hrpS*::Tn*5-gusA1*. Error bars: standard deviation for three replicates. Cells grown in inducing medium (IM) were assayed (Materials and Methods contains details).

Table 4. Hypersensitive reaction (HR) elicitation by *hrp* regulation mutants

Strain	Genotype	HR phenotype[a]
Ea321	wild type; *hrp*+	+++
Ea321-P7	*hrpX*	±
Ea321-P7(pCPP1178)	*hrpX*(*hrpXY*+)	++[b]
Ea321-P7(pCPP1001)	*hrpX*(*hrpS*+)	+++
Ea321-P7(pCPP1078)	*hrpX*(*hrpL*+)	+++
Ea321-P4	*hrpY*	–
Ea321-P4(pCPP1178)	*hrpY*(*hrpXY*+)	++[b]
Ea321-P4(pCPP1001)	*hrpY*(*hrpS*+)	+++
Ea321-P4(pCPP1078)	*hrpY*(*hrpL*+)	+++
Ea321-G107	*hrpS*	–
Ea321-G107(pCPP1178)	*hrpS*(*hrpXY*+)	–
Ea321-G107(pCPP1001)	*hrpS*(*hrpS*+)	+++

[a] +++, full HR manifested by complete tissue collapse throughout infiltrated area; ++, reduced HR, which is spotty and often coalescing; ±, infrequent collapse and small spotty necreosis for HR-positive leaves; and –, no HR. Inoculum concentration was approximately 2 × 10^8 CFU per ml. Ratings (consensus of four plants) were made 36 h after inoculation.

[b] Full HR was observed at inoculum levels of ≥ 5 × 10^8 CFU per ml.

1995). Also, the BvgS/BvgA system was recently found to be involved in the regulation of the type III secretion in *Bordetella bronchiseptica* (Yuk et al. 1998). Among plant pathogens, HrpG of *Xanthomonas campestris* pv. *vesicatoria*, a homolog of response regulators, has been shown to regulate *hrpXv* and *hrpA* expression (Wengelnik et al. 1996).

The structure of the input domain of *E. amylovora* HrpX appears to be exceptional, compared with sensor proteins involved in other type III systems, which contain two transmembrane regions and a periplasmic domain. The closest homologs of *E. amylovora* HrpY are SirA and BvgA, both of which are RO_{III}-type regulators (Parkinson and Kofoid 1992), whereas *X. campestris* HrpG belongs to the RO_{II} type, which includes *Escherichia coli* CpxR and OmpR, *S. typhimurium* PhoP, and *Agrobacterium tumefaciens* VirG. Thus, at least two types of transmitter-receiver systems appear to have evolved for control of type III systems in response to environmental stimuli in hosts. Also, the two two-component systems identified in the plant pathogens *E. amylovora* and *X. campestris* fall into different communication groups.

HrpS and mechanism of gene regulation.

HrpS is a member of the σ^{54}-dependent, enhancer-binding protein family. Both *hrpS* and *rpoN* are required for transcription of *hrp* genes in *P. syringae* pathovars (Grimm et al. 1995; Xiao et al. 1994). WtsA (HrpS) of *E. stewartii* controls expression of *wtsB*, which also requires the presence of σ^{54} (Frederick et al. 1993). In *E. amylovora*, HrpS partially regulates *hrpL* expression (Wei and Beer 1995), and a sequence, TGGCAC-N_5-TTGC, that perfectly matches the –24/–12 promoter consensus sequence is found at the promoter region of *E. amylovora hrpL*. The *hrpS* gene of *E. amylovora*, but not *hrpS* of *P. syringae* pv. *phaseolicola*, can complement the *hrpS* mutation in *E. stewartii* (Frederick et al. 1993). The HrpS sequences of the two erwinias are highly similar, and even the upstream noncoding regions appear to be conserved, except for the insertion of a 484-bp sequence, reminiscent of an IS (insertion sequence) element, 23-bp upstream of the *E. stewartii hrpS* ORF.

As a member of the NtrC family, HrpS is unusual in that it lacks a long N-terminal receiver domain. Control of protein activation by phosphorylation, by protein-protein interaction, and by signal molecule have been suggested for σ^{54}-dependent proteins (Shingler 1996). In the direct activation model, derepression by effectors seems to be a mechanism of protein activation. For DctD, DmpR, and XylR, deletion of the receiver domain results in constitutive activation of the proteins, suggesting that the receiver domain has a repressor function

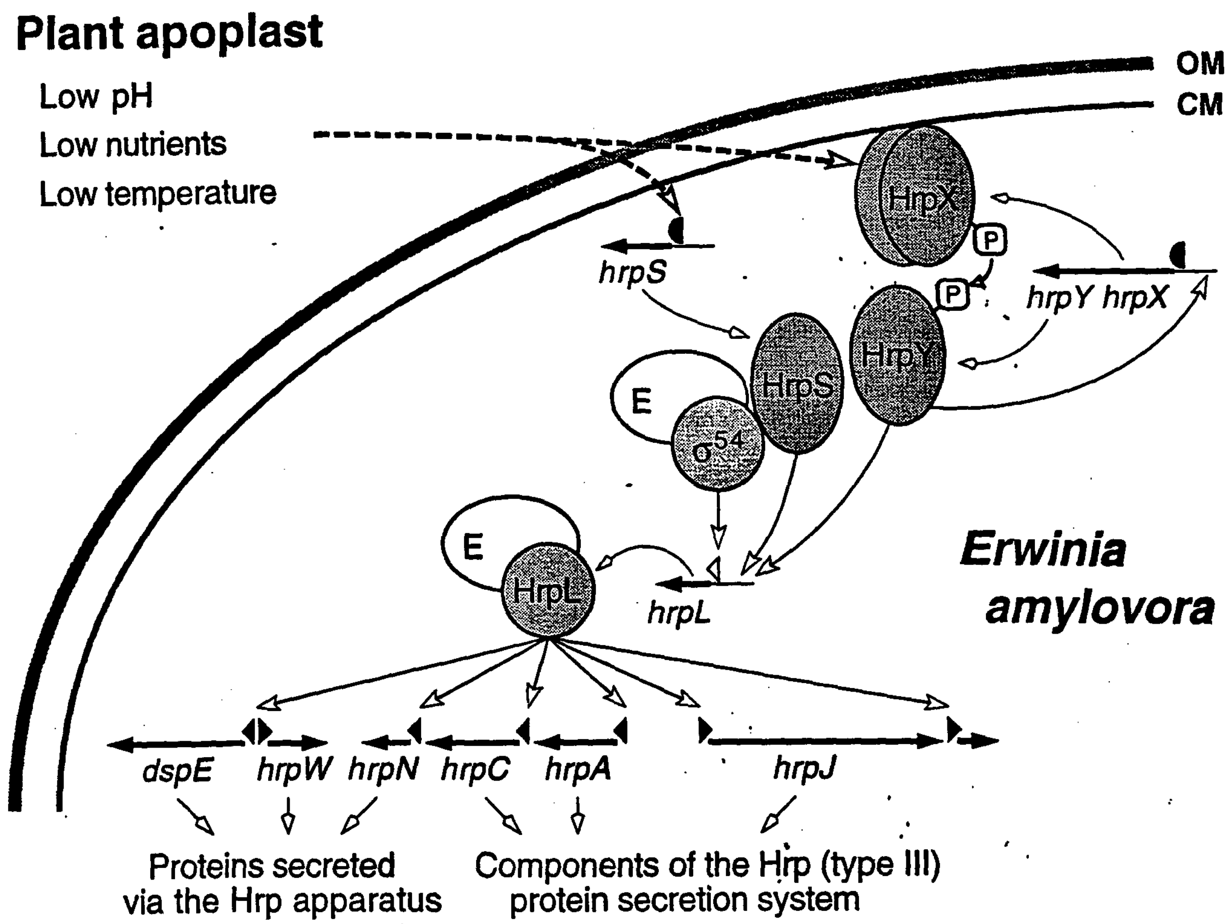


Fig. 7. Model of the *hrp* gene regulatory cascade. Thick arrow lines: genes or operons. Ovals and circles: proteins. Arrowheads in thinner lines: directions of information flow. CM, cytoplasmic membrane; OM, outer membrane; P, phosphate; E, RNA polymerase; closed half circle, σ^{70} promoter; open triangle, σ^{54} promoter; and filled triangle, HrpL promoter.

(Shingler 1996). Therefore, the apparent absence of the receiver domain in HrpS implies that HrpS may not require phosphorylation for activation and is always active once the protein is made.

Induction of *hrpXY* and *hrpS* and the involvement of HrpXY and HrpS in *hrpL* regulation.

Expression of *hrpS* and *hrpXY* is induced by conditions that mimic the apoplastic environment (Wei et al. 1992; this work). *hrpXY* shows high basal-level expression, and autoregulation is involved in gene induction. However, *hrpS* is not autoregulated based on results of the GUS assay, suggesting that there may be upstream regulatory components. Although *hrpS* provided in multiple-copy plasmids reverses the Hrp$^-$ phenotype of *hrpX* and *hrpY* mutants, the independence of *hrpS* from *hrpX* and *hrpY* suggests that *hrpXY* is not epistatic to *hrpS* and environmental signals may go to *hrpS* through a different pathway.

Earlier work on *hrpL* and *hrpS* (Wei and Beer 1995) established that HrpS partially controls *hrpL* expression. Our current work indicates that the HrpX/HrpY system contributes to *hrpL* induction. Based on the role of *hrpXY* and *hrpS* in regulating *hrpL* and the lack of effect of *hrpX* and *hrpY* in *hrpS* expression, one might place *hrpS* upstream of *hrpXY*. This notion is precluded, however, because *hrpXY* does not override *hrpS* mutation. As mentioned above, the opposite is not likely, either. Therefore, it seems that signals independently perceived by *hrpXY* and *hrpS* converge at *hrpL*.

Neither HrpS nor HrpY alone induce high levels of *hrpL* expression, suggesting that cooperation of HrpY and HrpS, possibly through protein-protein interaction, may be needed for full activation of *hrpL*. In this model, HrpS may be a positive activator of *hrpL*, while HrpX/HrpY may act as a modulator of *hrpL* transcription. Complementation of *hrpX* and *hrpY* mutants for the HR phenotype by overexpressed *hrpS* supports this model. The regulation of *eps* genes of *R. solanacearum* seems similar; both VsrD and PhcA regulators bind to the *xpsR* promoter region and control *xpsR* expression (Huang et al. 1995b). In *P. syringae* pv. *syringae*, HrpR and HrpS have been proposed to work together to control *hrpL* expression (Xiao et al. 1994), although a different opinion exists for homologous proteins in *P. syringae* pv. *phaseolicola* (Grimm et al. 1995).

***hrp* gene regulation and Hrp phenotypes.**

hrpY and *hrpS* seem to be crucial to the pathogenic life-style of *E. amylovora*, since their inactivation by mutagenesis results in loss of pathogenicity in immature pears (Wei et al. 1992; this work). The *hrpX* mutant, however, shows an attenuated phenotype: slightly lowered *hrpL* expression and reduced HR and virulence at higher inoculum doses. Currently, we cannot rule out the possibility of partial HrpX function in that mutant, even though leaky phenotypes of sensor mutants have been documented for other two-component systems (Stock et al. 1989). It is interesting to note that, although *hrpX* and *hrpS* mutants show different phenotypes (the former reduced Hrp and the latter Hrp$^-$), both are similarly affected in *hrpL* expression, i.e., only three- to fourfold reduction. This suggests that either there is a threshold level of *hrp* gene expression required for causing disease, or *hrpS* is involved in expression of other genes that contribute to pathogenicity. Further study might distinguish between these two possibilities.

The incomplete complementation of *hrpX* and *hrpY* mutants by *hrpXY* provided in a multicopy plasmid at lower inoculum levels ($\leq 2 \times 10^8$ CFU per ml) is intriguing and deserves further investigation. One explanation for the results could be that defective HrpX and HrpY in the mutants interact with functional HrpX and HrpY, and, possibly by forming heterodimers, interfere with the full activity. Alternatively, overproduced HrpX and HrpY may somehow down-regulate *hrpS* expression.

Model of the *E. amylovora hrp* gene expression.

Based on previous studies (Bogdanove et al. 1996, 1998b; Kim and Beer 1998; Kim et al. 1997; Wei and Beer 1995; Wei et al. 1992) and results described in this work, we propose a scheme of *hrp* gene regulation in *E. amylovora* (Fig. 7). When the bacteria enter the plant apoplast, HrpX perceives environmental signals and is phosphorylated. Activated HrpX then phosphorylates HrpY to activate it, and increases the expression of *hrpXY* to produce more HrpX and HrpY. Independently, expression of *hrpS* is induced in response to the changed environment. Activated HrpY and HrpS, bound to the *hrpL* promoter, then interact with the RNA polymerase-σ^{54} complex to drive transcription of *hrpL*. HrpS also activates other genes containing the –24/–12 promoter consensus sequence. Finally, the HrpL σ factor, which recognizes a conserved promoter motif, GGAACC-N_{15}-CCACTAAT, directs transcription of the remaining *hrp* and *dsp* genes that produce the secretion machinery and virulence proteins that interact with plant cells.

MATERIALS AND METHODS

Bacterial strains and growth condition.

E. amylovora Ea321 is a wild-type strain that infects pear and apple (Beer et al. 1991). *Escherichia coli* DH5α was routinely used for cloning of cosmids and plasmids. pCPP1001 (Wei and Beer 1995), pCPP1036 (Kim et al. 1997), pCPP1078 (Wei and Beer 1995), and pCPP1178 are subclones of pCPP430 (Beer et al. 1991), and contain ORFs in the same direction as the T7Φ10 promoter from the vector pBluescript KS+. Strains of *E. amylovora* Ea321 and *Escherichia coli* were grown in LB (Sambrook et al. 1989) with vigorous shaking at 28 and 37°C, respectively. Inducing medium (IM) was used for inducing *hrp* gene expression as described previously (Wei et al. 1992). The antibiotics used to maintain selection were ampicillin at 100 µg/ml, kanamycin (Km) at 50 µg/ml, spectinomycin (Sp) at 50 µg/ml, tetracycline (Tc) at 20 µg/ml, and carbenicillin (Cb) at 300 µg/ml.

Recombinant DNA techniques and sequence analysis.

Unless otherwise specified, basic molecular biology techniques were as described (Sambrook et al. 1989). Electroporation of plasmid DNA into *E. amylovora* 321 and its derivatives was done as described by Bauer and Beer (1991) with the Gene Pulser apparatus (Bio-Rad, Richmond, CA, U.S.A.).

Deletion clones, generated from the *ClaI-BglII* insert in pCPP1178 with the Erase-A-Base kit (Promega, Madison, WI, U.S.A.), were sequenced by the dideoxy chain termination procedure with the Sequenase sequencing kit (U.S. Biochemical, Cleveland, OH, U.S.A.). Also, sequencing of the region between *hrpA* and *hrpJ* in pCPP430, pCPP1001, pCPP1036, and pCPP1178 was performed on an ABI 373A automated DNA sequencer (Perkin-Elmer, Norwalk, CT, U.S.A.) at the

Cornell University Biotechnology Program DNA Sequencing Facility with oligonucleotide primers synthesized at the same facility.

DNA and deduced amino acid sequences were analyzed with programs in the GCG software package, version 7.3 (Genetics Computer Group, Madison, WI, U.S.A.) and DNASTAR (DNASTAR, Madison, WI, U.S.A.). Potential genes were identified with GeneMark.hmm (Lukashin and Borodovsky 1998; available on-line from the GeneMark web site). Homology searches were done with BLAST algorithms (Altschul et al. 1997; available on-line from the NCBI web site). Conserved patterns in proteins were found with ScanProsite (Appel et al. 1994; available on-line). Finally, prediction of potential σ^{70} promoters were made with the Promoter Prediction by Neural Network method (Reese and Eeckman 1995; available on-line).

Expression of *hrpX* and *hrpY* in *Escherichia coli*.

A gene expression system mediated by a T7 RNA polymerase/promoter (Tabor and Richardson 1985) was used. pCPP1178, which contains *hrpX* and *hrpY* ORFs driven by the T7Φ10 promoter from the vector, was introduced into *Escherichia coli* DH5α(pGP1-2). Cells were incubated at 42°C to induce the expression of the T7 RNA polymerase gene, and newly synthesized proteins were radiolabeled with ^{35}S-Met as described (Tabor and Richardson 1985). Resulting samples were resuspended in a sample buffer and heated to 95°C for 3 min before being electrophoresed in a 12% polyacrylamide gel.

Construction of marker-exchange mutants.

Chromosomal mutants were constructed by marker-exchange mutagenesis as described previously (Wei et al. 1992). A Tn*10*-miniKm insertion or a Tn*phoA* insertion, mapped at the *hrpXY* or *hrpL* locus in *Escherichia coli* DH5(pCCPP430) or *Escherichia coli* DH5α(pCPP1178), was introduced into *E. amylovora* Ea321 by triparental mating with the helper strain, *Escherichia coli* HB101(pRK600) (kindly provided by E. R. Signer; Department of Biology, Massachusetts Institute of Technology, Cambridge). The transconjugants were selected on Luria plates containing Km and Sp, and then transferred to a low-phosphate minimal medium (Bauer 1990) to select for Km^r Sp^s marker-exchanged mutants. The second mutations were generated by introducing individual *hrp*::Tn*5*-*gusA1* fusions into Tn*10*-miniKm or Tn*phoA* mutants of Ea321. Since the transposon Tn*5*-*gusA1* has two selection marker, Km and Tc, the second mutation was selected based on Km^r Tc^r Sp^s phenotype. All the mutants were tested for the HR-eliciting ability and pathogenicity. Tn*phoA* insertions P74 and P86 in pCPP1036, which were mapped to *orfU1*, were introduced to the Ea321 genome by electroporation and subsequent incubation in a low-phosphate medium with Km. Integration of the Tn*phoA* fusion into the chromosome was confirmed by antibiotic resistance (Km^r Cb^s) and Southern hybridization with the transposon DNA as a probe.

Assay of GUS activity.

Overnight cultures in LB were transferred to fresh LB, and incubated further. For incubation in IM, log-phase cultures in LB were centrifuged, and cells were washed with IM, before they are resuspended in IM to $OD_{620} \approx 0.5$. The cultures in IM were incubated for an additional 5 to 6 h at 24°C before assay of GUS activity. GUS activity was monitored fluorimetrically as described by Jefferson et al. (1987). Forty-five microliters of the log-phase culture in LB or the induced culture from IM was mixed with an equal volume of 2× assay buffer. After incubation at 37°C for 10 h, GUS activity was measured as described previously (Wei et al. 1992). The background fluorescence of Ea321-G77 (*hrcV*::Tn*5*-*gusA1*) (Wei et al. 1992), which has a *gusA1* insertion in the opposite direction of *hrcV* transcription, was subtracted from the readings of *hrp*::*gusA1* fusion strains. The corrected fluorescence readings were converted to picounits of GUS activity per CFU. The GUS activity of *hrp*::Tn*5*-*gusA1* fusions also were determined in tobacco leaf tissues as described previously (Wei et al. 1992).

Plant assays.

Bacteria were grown in LB and harvested at mid-exponential phase. Cells were resuspended in 5 mM potassium phosphate buffer, pH 6.5, harvested again, resuspended in the potassium phosphate buffer to approximately 2×10^8 CFU per ml, unless otherwise specified, and used for HR and pathogenicity assays. Tobacco plants (*Nicotiana tabacum* L. 'Xanthi') were grown in greenhouse soil mix to a height of 0.9 to 1 m. Bacterial suspensions were infiltrated into each leaf panel of tobacco leaves with needleless hypodermic syringes. The development of the HR was scored after incubation at room temperature for 18 to 36 h. Pathogenicity tests on immature pear fruits were carried out as previously described (Bauer and Beer 1991; Steinberger and Beer 1988).

ACKNOWLEDGMENTS

We acknowledge Barbara J. Sneath for her initial characterization of the *hrpS* locus, David W. Bauer for providing pCPP43, and Pakorn Kanchanawong for assisting with the epistasis experiment. We thank Adam J. Bogdanove, Stephen C. Winans, and anonymous reviewers for critical reading and suggestions. This work was supported by USDA CGRO grant 91-3-7303-6430, by USDA Special Research grant 99-34367-7990, by Eden Bioscience Corporation, Bothell, WA, and by the Cornell Center for Advanced Technology (CAT) in Biotechnology, which is sponsored by the New York State Science and Technology Foundation and industrial partners.

NOTE ADDED IN PROOF

A recent BLAST survey of finished and unfinished microbial genomes (available on-line from the NCBI web site) suggests the presence in *Pseudomonas aeruginosa* PAO1 of a two-component system that is highly similar to the HrpX/HrpY system (31% identity over 474 amino acids for HrpX and 48% identity over 208 amino acids for HrpY). A related set of proteins exist in the *Pseudomonas putida* KT2440 genome.

LITERATURE CITED

Alfano, J. R., and Collmer, A. 1996. Bacterial pathogens in plants: Life up against the wall. Plant Cell 8:1683-1698.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. L. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402.

Appel, R. D., Bairoch, A., and Hochstrasser, D. F. 1994. A new generation of information retrieval tools for biologists: The example of the

ExPASy WWW server. Trends Biochem. Sci. 19:258-260.
Bauer, D. W. 1990. Molecular genetics of pathogenicity of *Erwinia amylovora*: Techniques, tools and their applications. Ph.D. thesis. Cornell University, Ithaca, NY, U.S.A.
Bauer, D. W., and Beer, S. V. 1991. Further characterization of an *hrp* gene cluster of *Erwinia amylovora*. Mol. Plant-Microbe Interact. 4:493-499.
Beer, S. V., Bauer, D. W., Jiang, X. H., Laby, R. J., Sneath, B. J., Wei, Z.-M., Wilcox, D. A., and Zumoff, C. H. 1991. The *hrp* gene cluster of *Erwinia amylovora*. Pages 53-60 in: Advances in Molecular Genetics of Plant-Microbe Interactions, Vol. 1. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
Bogdanove, A. J., Bauer, D. W., and Beer, S. V. 1998a. *Erwinia amylovora* secretes DspE, a pathogenicity factor and functional AvrE homolog, through the Hrp (type III secretion) pathway. J. Bacteriol. 180:2244-2247.
Bogdanove, A. J., Kim, J. F., Wei, Z., Kolchinsky, P., Charkowski, A. O., Conlin, A. K., Collmer, A., and Beer, S. V. 1998b. Homology and functional similarity of a *hrp*-linked pathogenicity operon, *dspEF*, of *Erwinia amylovora* and the *avrE* locus of *Pseudomonas syringae* pathovar tomato. Proc. Natl. Acad. Sci. U.S.A. 95:1325-1330.
Bogdanove, A. J., Wei, Z.-M., Zhao, L., and Beer, S. V. 1996. *Erwinia amylovora* secretes harpin via a type III pathway and contains a homolog of *yopN* of *Yersinia*. J. Bacteriol. 178:1720-1730.
Eriksson, A. R. B., Andersson, R. A., Pirhonen, M., and Palva, E. T. 1998. Two-component regulators involved in the global control of virulence in *Erwinia carotovora* subsp. *carotovora*. Mol. Plant-Microbe Interact. 11:743-752.
Frederick, R. D., Majerczak, D. R., and Coplin, D. L. 1993. *Erwinia stewartii* WtsA, a positive regulator of pathogenicity gene expression, is similar to *Pseudomonas syringae* pv. *phaseolicola* HrpS. Mol. Microbiol. 9:477-485.
Friedrich, M. J., and Kadner, R. J. 1987. Nucleotide sequence of the *uhp* region of *Escherichia coli*. J. Bacteriol. 169:3556-3563.
Galán, J. E., and Bliska, J. B. 1996. Cross-talk between bacterial pathogens and their host cells. Annu. Rev. Cell. Dev. Biol. 12:221-255.
Gaudriault, S., Malandrin, L., Paulin, J.-P., and Barny, M.-A. 1997. DspA, an essential pathogenicity factor of *Erwinia amylovora* showing homology with AvrE of *Pseudomonas syringae*, is secreted via the Hrp secretion pathway in a DspB-dependent way. Mol. Microbiol. 26:1057-1069.
Goodman, R. N., and Novacky, A. J. 1994. The Hypersensitive Reaction in Plants to Pathogens: A Resistance Phenomenon. American Phytopathological Society, St. Paul, MN.
Grimm, C., Aufsatz, W., and Panopoulos, N. J. 1995. The *hrpRS* locus of *Pseudomonas syringae* pv. *phaseolicola* constitutes a complex regulatory unit. Mol. Microbiol. 15:155-165.
Hoch, J. A., and Silhavy, T. J. 1995. Two-component signal transduction. American Society for Microbiology, Washington, DC, U.S.A.
Hong, K. H., and Miller, V. L. 1998. Identification of a novel *Salmonella* invasion locus homologous to *Shigella ipgDE*. J. Bacteriol. 180:1793-1802.
Huang, H.-C., Lin, R.-H., Chang, C.-J., Collmer, A., and Deng, W. L. 1995a. The complete *hrp* gene cluster of *Pseudomonas syringae* pv. *syringae* 61 includes two blocks of genes required for harpin$_{Pss}$ secretion that are arranged colinearly with *Yersinia ysc* homologs. Mol. Plant-Microbe Interact. 8:733-746.
Huang, J., Carney, B. F., Denny, T. P., Weissinger, A. K., and Schell, M. A. 1995b. A complex network regulates expression of *eps* and other virulence genes of *Pseudomonas solanacearum*. J. Bacteriol. 177:1259-1267.
Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. 1987. GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901-3907.
Jiang, J., Gu, B. H., Albright, L. M., and Nixon, B. T. 1989. Conservation between coding and regulatory elements of *Rhizobium meliloti* and *Rhizobium leguminosarum dct* genes. J. Bacteriol. 171:5244-5253.
Johnston, C., Peques, D. A., Hueck, C. J., Lee, C. A., and Miller, S. I. 1996. Transcriptional activation of *Salmonella typhimurium* invasion genes by a member of the phosphorylated response-regulator superfamily. Mol. Microbiol. 22:715-727.
Kay, S. A. 1997. PAS, present, and future: Clues to the origins of circadian clocks. Science 276:753-754.
Kim, J. F., and Beer, S. V. 1998. HrpW of *Erwinia amylovora*, a new harpin that contains a domain homologous to pectate lyases of a distinct class. J. Bacteriol. 180:5203-5210.
Kim, J. F., Wei, Z.-M., and Beer, S. V. 1997. The *hrpA* and *hrpC* operons of *Erwinia amylovora* encode components of a type III pathway that secretes harpin. J. Bacteriol. 179:1690-1697.
Kunst, F., Debarbouille, M., Msadek, T., Young, M., Mauel, C., Karamata, D., Klier, A., Rapoport, G., and Dedonder, R. 1988. Deduced polypeptides encoded by the *Bacillus subtilis sacU* locus share homology with two-component sensor-regulator systems. J. Bacteriol. 170:5093-5101.
Larsen, J. E. L., Gerdes, K., Light, J., and Molin, S. 1984. Low-copy-number plasmid-cloning vectors amplifiable by derepression of an inserted foreign promoter. Gene 28:45-54.
Laville, J., Voisard, C., Keel, C., Maurhofer, M., Defago, G., and Haas, D. 1992. Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. Proc. Natl. Acad. Sci. U.S.A. 89:1562-1566.
Lonetto, M. A., Brown, K. L., Rudd, K. E., and Buttner, M. J. 1994. Analysis of the *Streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial RNA polymerase s factors involved in the regulation of extracytoplasmic functions. Proc. Natl. Acad. Sci. U.S.A. 91:7573-7577.
Lukashin, A. V., and Borodovsky, M. 1998. GeneMark.hmm: New solution for gene finding. Nucleic Acids Res. 26:1107-1115.
Morett, E., and Segovia, L. 1993. The s^{54} bacterial enhancer-binding protein family: Mechanisms of action and phylogenetic relationship of their functional domains. J. Bacteriol. 175:6067-6074.
Nakayama, S.-I., and Watanabe, H. 1995. Involvement of *cpxA*, a sensor of a two-component regulatory system, in the pH-dependent regulation of expression of *Shigella sonnei virF* gene. J. Bacteriol. 177:5062-5069.
Parkinson, J. S., and Kofoid, E. C. 1992. Communication modules in bacterial signaling proteins. Annu. Rev. Genet. 26:71-112.
Pegues, D. A., Hantmana, M. J., Behlau, I., and Miller, S. I. 1995. PhoP/PhoQ transcriptional repression of *Salmonella typhimurium* invasion genes: Evidence for a role in protein secretion. Mol. Microbiol. 17:169-181.
Rabin, R. S., and Stewart, V. 1993. Dual response regulators (NarL and NarP) interact with dual sensors (NarX and NarQ) to control nitrate- and nitrite-regulated gene expression in *Escherichia coli* K-12. J. Bacteriol. 175:3259-3268.
Reese, M. G., and Eeckman, F. H. 1995. New neural network algorithms for improved eukaryotic promoter site recognition. Genome Sci. Tech. 1:45-46.
Ronson, C. W., Astwood, P. M., Nixon, B. T., and Ausubel, F. M. 1987. Deduced products of C4-dicarboxylate transport regulatory genes of *Rhizobium leguminosarum* are homologous to nitrogen regulatory gene products. Nucleic Acids Res. 15:7921-7934.
Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.
Sharma, S., Stark, T. F., Beattie, W. G., and Moses, R. E. 1986. Multiple control elements for the *uvrC* gene unit of *Escherichia coli*. Nucleic Acids Res. 14:2301-2318.
Shingler, V. 1996. Signal sensing by s^{54}-dependent regulators: Derepression as a control mechanism. Mol. Microbiol. 19:409-416.
Sneath, B. J., Howson, J. M., and Beer, S. V. 1990. A pathogenicity gene from *Erwinia amylovora* encodes a predicted protein product homologous to a family of procaryotic response regulators. (Abstr.) Phytopathology 80:1038.
Steinberger, E. M., and Beer, S. V. 1988. Creation and complementation of pathogenicity mutants of *Erwinia amylovora*. Mol. Plant-Microbe Interact. 1:135-144.
Stock, J. B., Ninfa, A. J., and Stock, A. M. 1989. Protein phosphorylation and regulation of adaptive response in bacteria. Microbiol. Rev. 53:450-490.
Tabor, S., and Richardson, C. C. 1985. A bacteriophage T7 DNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. U.S.A. 82:1074-1078.
van der Zwet, T., and Beer, S. V. 1999. Fire blight—Its nature, prevention and control: A practical guide to integrated disease management. Agric. Information Bull. 681, U.S. Department of Agriculture, Washington, DC, U.S.A.
Wei, Z.-M., and Beer, S. V. 1993. HrpI of *Erwinia amylovora* functions in secretion of harpin and is a member of a new protein family. J.

Bacteriol. 175:7958-7967.
Wei, Z.-M., and Beer, S. V. 1995. *hrpL* activates *Erwinia amylovora hrp* gene transcription and is a member of the ECF subfamily of s factors. J. Bacteriol. 177:6201-6210.
Wei, Z.-M., Sneath, B. J., and Beer, S. V. 1992. Expression of *Erwinia amylovora hrp* genes in response to environmental stimuli. J. Bacteriol. 174:1875-1882.
Wengelnik, K., Van den Ackerveken, G., and Bonas, U. 1996. HrpG, a key *hrp* regulatory protein of *Xanthomonas campestris* pv. *vesicatoria* is homologous to two-component response regulators. Mol. Plant-Microbe Interact. 9:704-712.
Xiao, Y., Heu, S., Yi, J., Lu, Y., and Hutcheson, S. W. 1994. Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes. J. Bacteriol. 176:1025-1036.
Xiao, Y., and Hutcheson, S. W. 1994. A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae*. J. Bacteriol. 176:3089-3091.
Yuk, M. H., Harvill, E. T., and Miller, J. F. 1998. The BvgAS virulence control system regulates type III secretion in *Bordetella bronchiseptica*. Mol. Microbiol. 28:945-959.
Zhulin, I. B., Taylor, B. L., and Dixon, R. 1997. PAS domain S-boxes in Archaea, bacteria and sensors for oxygen and redox. Trends Biochem. Sci. 22:331-333.

 AC 2814

1994 Trends Microbiol 2:1-2

Bacterial home goal by harpins

Ulla Bonas

Host-pathogen interactions are dynamic and multifactorial; whether a microorganism succeeds or fails in colonizing a potential host depends on factors from both organisms. A successful pathogen has to overcome the defenses of the host. In bacteria that are pathogenic for animals or for plants, particularly Gram-negative organisms, a large number of genes are essential to infect host tissue and establish disease. Expression of these genes is generally controlled by environmental conditions such as temperature, pH, salt concentration and nutrient availability[1,2].

Pathogenicity, hypersensitive reaction and elicitors

In the Gram-negative plant pathogens *Erwinia*, *Pseudomonas* and *Xanthomonas*, genes organized in clusters of 25–40 kb are fundamentally involved in any obvious interaction with a plant (for a review see Ref. 3). These genes have been designated *hrp* (hypersensitive reaction and pathogenicity) because they are essential not only for pathogenicity towards a susceptible host plant, but also for interaction with resistant host varieties and with plants that are not a host for that pathogen. In plants, the hypersensitive reaction (HR) (Ref. 4) is a rapid defense reaction involving localized plant cell death and production of substances such as phenolics and phytoalexins at the site of infection. The HR prevents pathogen spread and thus halts disease development.

In the wild, plants are resistant to the majority of pathogens. The HR, therefore, is an important defense mechanism against all kinds of possible disease agents (bacteria, fungi, nematodes and viruses). It is not only important to interactions of pathogens with nonhost plants, but also to interactions between plants that carry resistance genes and microorganisms that are pathogens for that species.

Although the genes involved in plant defense[5,6] are becoming better understood, very little is known about the nature of the initial signals and their perception. Induction of the HR in a bacterium–plant interaction requires functional *hrp* genes and appears to be mediated by signal molecules or 'elicitors'. Recent DNA sequence analyses indicate that several putative Hrp proteins from different species are related and may be involved in a secretion system reminiscent of secretion of Yops (*Yersinia* outer proteins) in *Yersinia*[7–11]. So far, only one specific elicitor of the HR in a bacterium–plant interaction has been described. The *avrD* gene from *Pseudomonas syringae* pv. *tomato* mediates production of a low-molecular-mass compound that specifically induces the HR only in the soybean plant (a nonhost) when it carries the corresponding *Rpg4* resistance gene[5,12].

Harpins

Recently, two bacterial HR-inducing proteins, called 'harpins', were identified in *Erwinia amylovora*[13] and *P. syringae* pv. *syringae*[14]. Although the harpins differ in primary sequence, they have several features in common: they are glycine rich and heat stable, and they both induce an HR in tobacco, a nonhost plant for these bacteria. The genes encoding harpins are localized within the *hrp* clusters and obviously have a dual role in that they are also required for pathogenicity towards the normal host plant. Both *hrp* clusters allow nonpathogenic bacteria, such as *Escherichia coli*, to induce an HR in tobacco after recombinant expression, suggesting that the genes for the tobacco HR elicitors are present within the clusters[15,16].

U. Bonas is in the CNRS-Institut des Sciences Végétales, Avenue de la Terrasse, F-91198 Gif-sur-Yvette, France.

The first harpin to be identified, harpin$_{Ea}$, is a cell-envelope-associated protein encoded by the *hrpN* gene of *Er. amylovora*, a pathogen of pear and apple[13]. Recently, He and co-workers[14] have used an elegant approach to identify harpin$_{Pss}$, which is encoded by the *hrpZ* gene in the bean pathogen *P. s.* pv. *syringae*. Lysates of an expression library in *E. coli*, made using the cloned *P. s.* pv. *syringae hrp* cluster, were directly screened for HR-inducing activity on tobacco leaves. Two proteins were identified, one of which was an amino-terminal deletion of harpin$_{Pss}$ with even higher activity than the full-size protein; whether processing occurs during natural infection is not clear. Interestingly, the carboxyl terminus contains two short, direct repeats that are essential for elicitor activity. The activity is in the same range as that of the *Erwinia* harpin$_{Ea}$; however, to elicit an HR in other plants requires higher levels of the elicitor. He *et al.* show convincingly that the secretion of harpin$_{Pss}$ by *P. s.* pv. *syringae* depends on a product called HrpH that is closely related to proteins in other plant pathogens, and also in animal pathogens such as *Yersinia* and *Shigella*, where they are essential for protein secretion[9,10,14].

These exciting findings help verify the model that Hrp proteins are involved in the transport of elicitors and virulence factors[7]. Not surprisingly, the results presented by He and co-workers[14] also stimulate many questions. It needs to be shown that harpin$_{Pss}$ is actually secreted when the bacterium interacts with tobacco tissue (the *hrp* genes were induced *in vitro*). The concentrati n needed for HR induction (more than 600 nM) is much higher than one would expect for specific signal molecules. Are harpins toxins? Most importantly, what is their function in pathogenicity, and why do they

not elicit an HR in the host plant? Are harpins the only elicitors of nonhost HR in tobacco and possibly in other plants? Is the same mechanism used in tobacco to recognize both the *Erwinia* and the *P. s.* pv. *syringae* harpins? Is host resistance different in mechanism from nonhost resistance? Answers to this fascinating puzzle require the identification of more HR elicitors and their putative plant receptors.

References

1 Mekalanos, J.J. (1992) *J. Bacteriol.* 174, 1–7
2 Long, S.R. and Staskawicz, B. (1993) *Cell* 73, 921–935
3 Willis, D.K., Rich J.J. and Hrabak, E.M. (1991) *Mol. Plant–Microbe Interact.* 4, 132–138
4 Klement, Z. (1982) in *Phytopathogenic Prokaryotes* (Vol. 2) (Mount, M.S. and Lacy, G.H., eds), pp. 149–177, Academic Press
5 Keen, N.T. (1992) *Plant Mol. Biol.* 19, 109–122
6 Lindsay, W.P., Lamb, C.J. and Dixon, R.A. (1993) *Trends Microbiol.* 1, 181–187
7 Fenselau, S., Balbo, I. and Bonas, U. (1992) *Mol. Plant–Microbe Interact.* 5, 390–396
8 Gough, C.L. *et al.* (1992) *Mol. Plant–Microbe Interact.* 5, 384–389
9 Huang, H-C. *et al.* (1992) *J. Bacteriol.* 174, 6878–6885
10 Van Gijsegem, F., Genin, S. and Boucher, C. (1993) *Trends Microbiol.* 1, 175–180
11 Huang, H-C. *et al.* (1993) *Mol. Plant–Microbe Interact.* 6, 515–520
12 Smith, M.J. *et al.* (1993) *Tetrahedron Lett.* 34, 223–226
13 Wei, Z-M. *et al.* (1992) *Science* 257, 85–88
14 He, S.Y., Huang, H-C. and Collmer, A. (1993) *Cell* 73, 1255–1266
15 Huang, H-C. *et al.* (1988) *J. Bacteriol.* 170, 4748–4756
16 Beer, S.V. *et al.* (1991) in *Advances in Molecular Genetics of Plant–Microbe Interactions* (Hennecke, H. and Verma, D.P.S., eds), pp. 53–60, Kluwer Academic Publishers

Initiation and spread of α-herpesvirus infections

Thomas C. Mettenleiter

Herpesviruses are large animal viruses with a DNA genome varying from approximately 120 to 250 kb. Based on their biological properties, the Herpesviridae have been divided into three subfamilies, the α-, β- and γ-herpesvirinae, prototypes of which are the human pathogens herpes simplex virus (HSV), cytomegalovirus (HCMV) and Epstein–Barr virus (EBV), respectively. As enveloped viruses, they depend on two consecutive processes for infectious entry into target cells: (1) attachment of free virions to cells and (2) penetration, that is, fusion of virion envelope and cellular cytoplasmic membrane leading to release of the nucleocapsid into the cell. Virion envelope glycoproteins play important roles in both these processes (see Refs 1,2 for recent reviews).

After infection of primary target cells, virus spread can occur by several different mechanisms. Infected cells may release infectious virions that reinitiate infection from outside. In addition, direct viral cell-to-cell spread from primary infected cells to adjacent noninfected cells may occur. In the host, virus may be disseminated by circulating infected cells that adhere to noninfected tissues and transmit infectivity directly. Recent results on HSV and pseudorabies virus (PrV) shed more light on these processes in α-herpesviruses. PrV causes Aujeszky's disease in swine, which is characterized by nervous and respiratory symptoms, and reproductive failure. Unlike HSV, PrV is not pathogenic for humans. However, the two viruses have several features in common, including a broad host range *in vitro*, and several species besides the natural host can be infected experimentally. In addition, all of the known PrV glycoproteins are related to homologous glycoproteins in HSV (Ref. 1)*.

T.C. Mettenleiter is in the Federal Research Centre for Virus Diseases of Animals, PO Box 1149, D-72001 Tübingen, Germany.

Attachment

Binding of free infectious virus to target cells involves interactions between virion envelope glycoproteins and cellular virus receptors. Herpes virions contain a large number of different virus-encoded envelope glycoproteins that might participate in attachment. A well-known example of a cellular herpesvirus receptor is the B-cell membrane protein CR2 (CD21), which binds EBV (Ref. 3). Recent studies have demonstrated that several α- (reviewed in Ref. 1), β- and γ-herpesviruses[4,5] bind to their target cells by interaction of virion components with cell-surface glycosaminoglycans, principally heparan sulfate (HS)[6].

*At the 18th International Herpesvirus Workshop, a common nomenclature for α-herpesvirus glycoproteins was agreed on, based on designations of HSV glycoproteins. This nomenclature is used here.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 December 2001 (27.12.2001)

PCT

(10) International Publication Number
WO 01/98501 A2

(51) International Patent Classification[7]: C12N 15/31, C07K 14/195, C12N 15/62, 15/82, A01H 5/00

(21) International Application Number: PCT/US01/18820

(22) International Filing Date: 12 June 2001 (12.06.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/212,211 16 June 2000 (16.06.2000) US

(71) Applicant: EDEN BIOSCIENCE CORPORATION [US/US]; 11816 Nnorth Creek Parkway N., Bothell, WA 98011-8205 (US).

(72) Inventors: FAN, Hao; 19712 6th Drive S.E., Bothell, WA 98012 (US). WEI, Zhong-Min; 8230 125th Court, Kirkland, WA 98034 (US).

(74) Agents: GOLDMAN, Michael, L. et al.; Nixon Peabody LLP, Clinton Square, P.O. Box 31051, Rochester, NY 14603-1051 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: HYPERSENSITIVE RESPONSE ELICITING DOMAINS AND USE THEREOF

(57) Abstract: The present invention is directed to the structure of an isolated protein or polypeptide which elicits a hypersensitive response in plants as well as an isolated nucleic acid molecule which encodes the hypersensitive response eliciting protein or polypeptide. This protein or polypeptide has an acid portion linked to an alpha helix or a pair of spaced apart domains comprising an acidic portion linked to an alpha-helix. This isolated protein or polypeptide and the isolated nucleic acid molecule can be used to impart disease resistance to plants, to enhance plant growth, to control insects, and/or to impart stress resistance to plants. This can be achieved by applying the hypersensitive response elicitor protein or polypeptide in a non-infectious form to plants or plant seeds under conditions effective to impart disease resistance, to enhance plant growth, to control insects, and/or to impart stress resistance to plants or plants grown from the plant seeds. Alternatively, transgenic plants or plant seeds transformed with a nucleic acid molecule encoding a hypersensitive response elicitor protein or polypeptide can be provided and the transgenic plants or plants resulting from the transgenic plant seeds are grown under conditions effective to impart disease resistance, to enhance plant growth, to control insects, and/or to impart stress resistance to plants or plants grown from the plant seeds.

WO 01/98501 A2

HYPERSENSITIVE RESPONSE ELICITING DOMAINS AND USE THEREOF

This application claims benefit of U.S. Provisional Patent Application Serial No. 60/212,211, filed on June 16, 2000.

FIELD OF THE INVENTION

The present invention relates to hypersensitive response elicitors and their structure.

BACKGROUND OF THE INVENTION

Interactions between bacterial pathogens and their plant hosts generally fall into two categories: (1) compatible (pathogen-host), leading to intercellular bacterial growth, symptom development, and disease development in the host plant; and (2) incompatible (pathogen-nonhost), resulting in the hypersensitive response, a particular type of incompatible interaction occurring, without progressive disease symptoms. During compatible interactions on host plants, bacterial populations increase dramatically and progressive symptoms occur. During incompatible interactions, bacterial populations do not increase, and progressive symptoms do not occur.

The hypersensitive response is a rapid, localized necrosis that is associated with the active defense of plants against many pathogens (Kiraly, Z., "Defenses Triggered by the Invader: Hypersensitivity," pages 201-224 in: Plant Disease: An Advanced Treatise, Vol. 5, J.G. Horsfall and E.B. Cowling, ed. Academic Press New York (1980); Klement, Z., "Hypersensitivity," pages 149-177 in: Phytopathogenic Prokaryotes, Vol. 2, M.S. Mount and G.H. Lacy, ed. Academic Press, New York (1982)). The hypersensitive response elicited by bacteria is readily observed as a tissue collapse if high concentrations ($\geq 10^7$ cells/ml) of a limited host-range pathogen like *Pseudomonas syringae* or *Erwinia amylovora* are infiltrated into the leaves of nonhost plants (necrosis occurs only in isolated plant cells at lower levels of inoculum) (Klement, Z., "Rapid Detection of Pathogenicity of Phytopathogenic Pseudomonads," Nature 199:299-300; Klement, et al.,

"Hypersensitive Reaction Induced by Phytopathogenic Bacteria in the Tobacco Leaf," Phytopathology 54:474-477 (1963); Turner, et al., "The Quantitative Relation Between Plant and Bacterial Cells Involved in the Hypersensitive Reaction," Phytopathology 64:885-890 (1974); Klement, Z., "Hypersensitivity," pages 149-177 in Phytopathogenic Prokaryotes, Vol. 2., M.S. Mount and G.H. Lacy, ed. Academic Press, New York (1982)). The capacities to elicit the hypersensitive response in a nonhost and be pathogenic in a host appear linked. As noted by Klement, Z., "Hypersensitivity," pages 149-177 in Phytopathogenic Prokaryotes, Vol. 2., M.S. Mount and G.H. Lacy, ed. Academic Press, New York, these pathogens also cause physiologically similar, albeit delayed, necroses in their interactions with compatible hosts. Furthermore, the ability to produce the hypersensitive response or pathogenesis is dependent on a common set of genes, denoted *hrp* (Lindgren, P.B., et al., "Gene Cluster of *Pseudomonas syringae* pv. 'phaseolicola' Controls Pathogenicity of Bean Plants and Hypersensitivity on Nonhost Plants," J. Bacteriol. 168:512-22 (1986); Willis, D.K., et al., "*hrp* Genes of Phytopathogenic Bacteria," Mol. Plant-Microbe Interact. 4:132-138 (1991)). Consequently, the hypersensitive response may hold clues to both the nature of plant defense and the basis for bacterial pathogenicity.

The *hrp* genes are widespread in gram-negative plant pathogens, where they are clustered, conserved, and in some cases interchangeable (Willis, D.K., et al., "*hrp* Genes of Phytopathogenic Bacteria," Mol. Plant-Microbe Interact. 4:132-138 (1991); Bonas, U., "*hrp* Genes of Phytopathogenic Bacteria," pages 79-98 in: Current Topics in Microbiology and Immunology: Bacterial Pathogenesis of Plants and Animals - Molecular and Cellular Mechanisms, J.L. Dangl, ed. Springer-Verlag, Berlin (1994)). Several *hrp* genes encode components of a protein secretion pathway similar to one used by *Yersinia, Shigella*, and *Salmonella* spp. to secrete proteins essential in animal diseases (Van Gijsegem, et al., "Evolutionary Conservation of Pathogenicity Determinants Among Plant and Animal Pathogenic Bacteria," Trends Microbiol. 1:175-180 (1993)). In *E. amylovora, P. syringae*, and *P. solanacearum*, hrp genes have been shown to control the production and secretion of glycine-rich, protein elicitors of the hypersensitive response (He, S.Y., et al. "Pseudomonas Syringae pv. Syringae HarpinPss: a Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," Cell 73:1255-1266 (1993), Wei, Z.-H.,

et al., "HrpI of *Erwinia amylovora* Functions in Secretion of Harpin and is a Member of a New Protein Family," J. Bacteriol. 175:7958-7967 (1993); Arlat, M. et al. "PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-553 (1994)).

The first of these proteins was discovered in *E. amylovora* Ea321, a bacterium that causes fire blight of rosaceous plants, and was designated harpin (Wei, Z.-M., et al, "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (1992)). Mutations in the encoding *hrpN* gene revealed that harpin is required for *E. amylovora* to elicit a hypersensitive response in nonhost tobacco leaves and incite disease symptoms in highly susceptible pear fruit. The *P. solanacearum* GMI1000 PopA1 protein has similar physical properties and also elicits the hypersensitive response in leaves of tobacco, which is not a host of that strain (Arlat, et al. "PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-53 (1994)). However, *P. solanacearum popA* mutants still elicit the hypersensitive response in tobacco and incite disease in tomato. Thus, the role of these glycine-rich hypersensitive response elicitors can vary widely among gram-negative plant pathogens.

Other plant pathogenic hypersensitive response elicitors have been isolated, cloned, and sequenced. These include: *Erwinia chrysanthemi* (Bauer, et. al., "*Erwinia chrysanthemi* Harpin$_{Ech}$: Soft-Rot Pathogenesis," MPMI 8(4): 484-91 (1995)); *Erwinia carotovora* (Cui, et. al., "The RsmA$^-$ Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrp*N$_{Ecc}$ and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI 9(7): 565-73 (1966)); *Erwinia stewartii* (Ahmad, et. al., "Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," 8th Int'l. Cong. Molec. Plant-Microb. Inter. July 14-19, 1996 and Ahmad, et. al., "Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," Ann. Mtg. Am. Phytopath. Soc. July 27-31, 1996); and *Pseudomonas syringae* pv. *syringae* (WO 94/26782 to Cornell Research Foundation, Inc.).

The present invention is a further advance in the effort to identify and characterize hypersensitive response elicitor proteins.

SUMMARY OF THE INVENTION

One aspect of the present invention is directed to an isolated hypersensitive response elicitor protein comprising a pair of spaced apart domains, with each comprising an acid portion linked to an alpha-helix.

Another embodiment of the present invention relates to an isolated hypersensitive response elicitor protein comprising an acid portion linked to an alpha-helix.

Nucleic acid molecules encoding either of these proteins as well as vectors, host cells, transgenic plants, and transgenic plant seeds containing those nucleic acid molecules are also disclosed.

The protein of the present invention can be used to impart disease resistance to plants, to enhance plant growth, to control insects, and/or impart stress resistance. This involves applying the protein to plants or plant seeds under conditions effective to impart disease resistance, to enhance plant growth, to control insects, and/or impart stress resistance to plants or plants grown from the plant seeds.

As an alternative to applying the protein to plants or plant seeds in order to impart disease resistance, to enhance plant growth, to control insects on plants, and/or impart stress resistance, transgenic plants or plant seeds can be utilized. When utilizing transgenic plants, this involves providing a transgenic plant transformed with a nucleic acid molecule encoding the protein of the present invention and growing the plant under conditions effective to impart disease resistance, to enhance plant growth, to control insects, and/or to impart stress resistance to the plants or plants grown from the plant seeds. Alternatively, a transgenic plant seed transformed with the nucleic acid molecule encoding the protein of the present invention can be provided and planted in soil. A plant is then propagated under conditions effective to impart disease resistance, to enhance plant growth, to control insects, and/or to impart stress resistance to plants or plants grown from the plant seeds.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic drawing showing the construction of a universal expression cassette for a hypersensitive response domain.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to an isolated hypersensitive response elicitor protein comprising a pair of spaced apart domains, with each comprising an acid portion linked to an alpha-helix. The acidic portion is a polypeptide with 10 or more amino acids, is rich in acidic amino acids, and has a pI below 5.0. The acidic portion has a secondary structure in the form of a beta-sheet or a beta-turn. The secondary structure of this unit can also be in an unordered form.

The alpha-helix portion of the present invention is a polypeptide with 10 or more amino acids. Its secondary structure is in the form of a stable alpha-helix.

Another embodiment of the present invention relates to an isolated hypersensitive response elicitor protein comprising an acid portion linked to an alpha-helix.

Both of these proteins are capable of eliciting a hypersensitive response.

The alpha helix is a common structural motif of proteins in which a linear sequence of amino acid folds into a right-handed helix stabilized by internal hydrogen bonding between backbone atoms.

The acidic motif includes a certain combination of amino acids in which a linear sequence with a pI below 5.0 folds into a β sheet, coil, or thin structures but not an alpha helix of secondary structure.

The hypersensitive response elicitor polypeptides or proteins according to the present invention can be derived from hypersensitive response elicitor polypeptides or proteins of a wide variety of fungal and bacterial pathogens. Such polypeptides or proteins are able to elicit local necrosis in plant tissue contacted by the elicitor. Examples of suitable bacterial sources of polypeptide or protein elicitors

include *Erwinia, Pseudomonas*, and *Xanthamonas* species (e.g., the following bacteria: *Erwinia amylovora, Erwinia chrysanthemi, Erwinia stewartii, Erwinia carotovora, Pseudomonas syringae, Pseudomonas solancearum, Xanthomonas campestris*, and mixtures thereof). In addition to hypersensitive response elicitors from these Gram negative bacteria, it is possible to use elicitors from Gram positive bacteria. One example is *Clavibacter michiganensis* subsp. *sepedonicus*.

An example of a fungal source of a hypersensitive response elicitor protein or polypeptide is *Phytophthora*. Suitable species of *Phytophthora* include *Phytophthora parasitica, Phytophthora cryptogea, Phytophthora cinnamomi, Phytophthora capsici, Phytophthora megasperma*, and *Phytophthora citrophthora*.

The hypersensitive response elicitor polypeptide or protein from *Erwinia chrysanthemi* has an amino acid sequence corresponding to SEQ. ID. No. 1 as follows:

```
Met Gln Ile Thr Ile Lys Ala His Ile Gly Gly Asp Leu Gly Val Ser
1               5                   10                  15

Gly Leu Gly Ala Gln Gly Leu Lys Gly Leu Asn Ser Ala Ala Ser Ser
            20                  25                  30

Leu Gly Ser Ser Val Asp Lys Leu Ser Ser Thr Ile Asp Lys Leu Thr
        35                  40                  45

Ser Ala Leu Thr Ser Met Met Phe Gly Gly Ala Leu Ala Gln Gly Leu
    50                  55                  60

Gly Ala Ser Ser Lys Gly Leu Gly Met Ser Asn Gln Leu Gly Gln Ser
65                  70                  75                  80

Phe Gly Asn Gly Ala Gln Gly Ala Ser Asn Leu Leu Ser Val Pro Lys
                85                  90                  95

Ser Gly Gly Asp Ala Leu Ser Lys Met Phe Asp Lys Ala Leu Asp Asp
            100                 105                 110

Leu Leu Gly His Asp Thr Val Thr Lys Leu Thr Asn Gln Ser Asn Gln
        115                 120                 125

Leu Ala Asn Ser Met Leu Asn Ala Ser Gln Met Thr Gln Gly Asn Met
    130                 135                 140

Asn Ala Phe Gly Ser Gly Val Asn Asn Ala Leu Ser Ser Ile Leu Gly
145                 150                 155                 160

Asn Gly Leu Gly Gln Ser Met Ser Gly Phe Ser Gln Pro Ser Leu Gly
                165                 170                 175
```

```
Ala Gly Gly Leu Gln Gly Leu Ser Gly Ala Gly Ala Phe Asn Gln Leu
            180                 185                 190

Gly Asn Ala Ile Gly Met Gly Val Gly Gln Asn Ala Ala Leu Ser Ala
        195                 200                 205

Leu Ser Asn Val Ser Thr His Val Asp Gly Asn Asn Arg His Phe Val
    210                 215                 220

Asp Lys Glu Asp Arg Gly Met Ala Lys Glu Ile Gly Gln Phe Met Asp
225                 230                 235                 240

Gln Tyr Pro Glu Ile Phe Gly Lys Pro Glu Tyr Gln Lys Asp Gly Trp
                245                 250                 255

Ser Ser Pro Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser Lys
            260                 265                 270

Pro Asp Asp Asp Gly Met Thr Gly Ala Ser Met Asp Lys Phe Arg Gln
        275                 280                 285

Ala Met Gly Met Ile Lys Ser Ala Val Ala Gly Asp Thr Gly Asn Thr
    290                 295                 300

Asn Leu Asn Leu Arg Gly Ala Gly Gly Ala Ser Leu Gly Ile Asp Ala
305                 310                 315                 320

Ala Val Val Gly Asp Lys Ile Ala Asn Met Ser Leu Gly Lys Leu Ala
                325                 330                 335

Asn Ala
```

This hypersensitive response elicitor polypeptide or protein has a molecular weight of 34 kDa, is heat stable, has a glycine content of greater than 16%, and contains substantially no cysteine. The *Erwinia chrysanthemi* hypersensitive response elicitor polypeptide or protein is encoded by a DNA molecule having a nucleotide sequence corresponding to SEQ. ID. No. 2 as follows:

```
CGATTTTACC CGGGTGAACG TGCTATGACC GACAGCATCA CGGTATTCGA CACCGTTACG     60
GCGTTTATGG CCGCGATGAA CCGGCATCAG GCGGCGCGCT GGTCGCCGCA ATCCGGCGTC    120
GATCTGGTAT TTCAGTTTGG GGACACCGGG CGTGAACTCA TGATGCAGAT TCAGCCGGGG    180
CAGCAATATC CCGGCATGTT GCGCACGCTG CTCGCTCGTC GTTATCAGCA GGCGGCAGAG    240
TGCGATGGCT GCCATCTGTG CCTGAACGGC AGCGATGTAT TGATCCTCTG GTGGCCGCTG    300
CCGTCGGATC CCGGCAGTTA TCCGCAGGTG ATCGAACGTT TGTTTGAACT GGCGGGAATG    360
ACGTTGCCGT CGCTATCCAT AGCACCGACG GCGCGTCCGC AGACAGGGAA CGGACGCGCC    420
CGATCATTAA GATAAAGGCG GCTTTTTTTA TTGCAAAACG GTAACGGTGA GGAACCGTTT    480
```

```
CACCGTCGGC GTCACTCAGT AACAAGTATC CATCATGATG CCTACATCGG GATCGGCGTG    540
GGCATCCGTT GCAGATACTT TTGCGAACAC CTGACATGAA TGAGGAAACG AAATTATGCA    600
AATTACGATC AAAGCGCACA TCGGCGGTGA TTTGGGCGTC TCCGGTCTGG GGCTGGGTGC    660
TCAGGGACTG AAAGGACTGA ATTCCGCGGC TTCATCGCTG GGTTCCAGCG TGGATAAACT    720
GAGCAGCACC ATCGATAAGT TGACCTCCGC GCTGACTTCG ATGATGTTTG GCGGCGCGCT    780
GGCGCAGGGG CTGGGCGCCA GCTCGAAGGG GCTGGGGATG AGCAATCAAC TGGGCCAGTC    840
TTTCGGCAAT GGCGCGCAGG GTGCGAGCAA CCTGCTATCC GTACCGAAAT CCGGCGGCGA    900
TGCGTTGTCA AAAATGTTTG ATAAAGCGCT GGACGATCTG CTGGGTCATG ACACCGTGAC    960
CAAGCTGACT AACCAGAGCA ACCAACTGGC TAATTCAATG CTGAACGCCA GCCAGATGAC   1020
CCAGGGTAAT ATGAATGCGT TCGGCAGCGG TGTGAACAAC GCACTGTCGT CCATTCTCGG   1080
CAACGGTCTC GGCCAGTCGA TGAGTGGCTT CTCTCAGCCT TCTCTGGGGG CAGGCGGCTT   1140
GCAGGGCCTG AGCGGCGCGG GTGCATTCAA CCAGTTGGGT AATGCCATCG GCATGGGCGT   1200
GGGGCAGAAT GCTGCGCTGA GTGCGTTGAG TAACGTCAGC ACCCACGTAG ACGGTAACAA   1260
CCGCCACTTT GTAGATAAAG AAGATCGCGG CATGGCGAAA GAGATCGGCC AGTTTATGGA   1320
TCAGTATCCG GAAATATTCG GTAAACCGGA ATACCAGAAA GATGGCTGGA GTTCGCCGAA   1380
GACGGACGAC AAATCCTGGG CTAAAGCGCT GAGTAAACCG GATGATGACG GTATGACCGG   1440
CGCCAGCATG GACAAATTCC GTCAGGCGAT GGGTATGATC AAAAGCGCGG TGGCGGGTGA   1500
TACCGGCAAT ACCAACCTGA ACCTGCGTGG CGCGGGCGGT GCATCGCTGG GTATCGATGC   1560
GGCTGTCGTC GGCGATAAAA TAGCCAACAT GTCGCTGGGT AAGCTGGCCA ACGCCTGATA   1620
ATCTGTGCTG GCCTGATAAA GCGGAAACGA AAAAAGAGAC GGGGAAGCCT GTCTCTTTTC   1680
TTATTATGCG GTTTATGCGG TTACCTGGAC CGGTTAATCA TCGTCATCGA TCTGGTACAA   1740
ACGCACATTT TCCCGTTCAT TCGCGTCGTT ACGCGCCACA ATCGCGATGG CATCTTCCTC   1800
GTCGCTCAGA TTGCGCGGCT GATGGGGAAC GCCGGGTGGA ATATAGAGAA ACTCGCCGGC   1860
CAGATGGAGA CACGTCTGCG ATAAATCTGT GCCGTAACGT GTTTCTATCC GCCCCTTTAG   1920
CAGATAGATT GCGGTTTCGT AATCAACATG GTAATGCGGT TCCGCCTGTG CGCCGGCCGG   1980
GATCACCACA ATATTCATAG AAAGCTGTCT TGCACCTACC GTATCGCGGG AGATACCGAC   2040
AAAATAGGGC AGTTTTTGCG TGGTATCCGT GGGGTGTTCC GGCCTGACAA TCTTGAGTTG   2100
GTTCGTCATC ATCTTTCTCC ATCTGGGCGA CCTGATCGGT T                       2141
```

The hypersensitive response elicitor from *Erwinia chrysanthemi* has 2 hypersensitive response eliciting domains. The first domain extends, within SEQ.

ID. No. 1, from amino acid 69 to amino acid 122, particularly from amino acid 85 to amino acid 116. The acidic unit in the first domain extends, within SEQ. ID. No. 1, from amino acid 69 to amino acid 102, particularly from amino acid 85 to amino acid 102. The alpha-helix in the first domain extends, within SEQ. ID. No. 1, from amino acid 102 to amino acid 122, particularly from amino acid 102 to amino acid 116. The second domain extends, within SEQ. ID. No. 1, from amino acid 251 to amino acid 299, particularly from amino acid 256 to amino acid 292. The acidic unit in the second domain extends, within SEQ. ID. No. 1, from amino acid 251 to amino acid 279, particularly from amino acid 261 to amino acid 279. The alpha-helix in the second domain extends, within SEQ. ID. No. 1, from amino acid 279 to amino acid 299, particularly from amino acid 279 to amino acid 292.

The hypersensitive response elicitor polypeptide or protein derived from *Erwinia amylovora* has an amino acid sequence corresponding to SEQ. ID. No. 3 as follows:

```
Met Ser Leu Asn Thr Ser Gly Leu Gly Ala Ser Thr Met Gln Ile Ser
1               5                   10                  15

Ile Gly Gly Ala Gly Gly Asn Asn Gly Leu Leu Gly Thr Ser Arg Gln
            20                  25                  30

Asn Ala Gly Leu Gly Gly Asn Ser Ala Leu Gly Leu Gly Gly Gly Asn
        35                  40                  45

Gln Asn Asp Thr Val Asn Gln Leu Ala Gly Leu Leu Thr Gly Met Met
    50                  55                  60

Met Met Met Ser Met Met Gly Gly Gly Gly Leu Met Gly Gly Gly Leu
65                  70                  75                  80

Gly Gly Gly Leu Gly Asn Gly Leu Gly Gly Ser Gly Gly Leu Gly Glu
                85                  90                  95

Gly Leu Ser Asn Ala Leu Asn Asp Met Leu Gly Gly Ser Leu Asn Thr
            100                 105                 110

Leu Gly Ser Lys Gly Gly Asn Asn Thr Thr Ser Thr Thr Asn Ser Pro
        115                 120                 125

Leu Asp Gln Ala Leu Gly Ile Asn Ser Thr Ser Gln Asn Asp Asp Ser
    130                 135                 140

Thr Ser Gly Thr Asp Ser Thr Ser Asp S r Ser Asp Pro Met Gln Gln
145                 150                 155                 160
```

Leu Leu Lys Met Phe Ser Glu Ile Met Gln Ser Leu Phe Gly Asp Gly
165 170 175

Gln Asp Gly Thr Gln Gly Ser Ser Ser Gly Gly Lys Gln Pro Thr Glu
180 185 190

Gly Glu Gln Asn Ala Tyr Lys Lys Gly Val Thr Asp Ala Leu Ser Gly
195 200 205

Leu Met Gly Asn Gly Leu Ser Gln Leu Leu Gly Asn Gly Gly Leu Gly
210 215 220

Gly Gly Gln Gly Gly Asn Ala Gly Thr Gly Leu Asp Gly Ser Ser Leu
225 230 235 240

Gly Gly Lys Gly Leu Gln Asn Leu Ser Gly Pro Val Asp Tyr Gln Gln
245 250 255

Leu Gly Asn Ala Val Gly Thr Gly Ile Gly Met Lys Ala Gly Ile Gln
260 265 270

Ala Leu Asn Asp Ile Gly Thr His Arg His Ser Ser Thr Arg Ser Phe
275 280 285

Val Asn Lys Gly Asp Arg Ala Met Ala Lys Glu Ile Gly Gln Phe Met
290 295 300

Asp Gln Tyr Pro Glu Val Phe Gly Lys Pro Gln Tyr Gln Lys Gly Pro
305 310 315 320

Gly Gln Glu Val Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser
325 330 335

Lys Pro Asp Asp Asp Gly Met Thr Pro Ala Ser Met Glu Gln Phe Asn
340 345 350

Lys Ala Lys Gly Met Ile Lys Arg Pro Met Ala Gly Asp Thr Gly Asn
355 360 365

Gly Asn Leu Gln Ala Arg Gly Ala Gly Gly Ser Ser Leu Gly Ile Asp
370 375 380

Ala Met Met Ala Gly Asp Ala Ile Asn Asn Met Ala Leu Gly Lys Leu
385 390 395 400

Gly Ala Ala

This hypersensitive response elicitor polypeptide or protein has a molecular weight of about 39 kDa, has a pI of approximately 4.3, and is heat stable at 100°C for at least 10 minutes. This hypersensitive response elicitor polypeptide or protein has substantially no cysteine. The hypersensitive response elicitor polypeptide or protein derived from *Erwinia amylovora* is more fully described in Wei, Z.-M., R. J. Laby, C. H. Zumoff,

D. W. Bauer, S.-Y. He, A. Collmer, and S. V. Beer, "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (1992), which is hereby incorporated by reference. The DNA molecule encoding this polypeptide or protein has a nucleotide sequence corresponding to SEQ. ID. No. 4 as follows:

```
AAGCTTCGGC ATGGCACGTT TGACCGTTGG GTCGGCAGGG TACGTTTGAA TTATTCATAA   60
GAGGAATACG TTATGAGTCT GAATACAAGT GGGCTGGGAG CGTCAACGAT GCAAATTTCT  120
ATCGGCGGTG CGGGCGGAAA TAACGGGTTG CTGGGTACCA GTCGCCAGAA TGCTGGGTTG  180
GGTGGCAATT CTGCACTGGG GCTGGGCGGC GGTAATCAAA ATGATACCGT CAATCAGCTG  240
GCTGGCTTAC TCACCGGCAT GATGATGATG ATGAGCATGA TGGGCGGTGG TGGGCTGATG  300
GGCGGTGGCT TAGGCGGTGG CTTAGGTAAT GGCTTGGGTG GCTCAGGTGG CCTGGGCGAA  360
GGACTGTCGA ACGCGCTGAA CGATATGTTA GGCGGTTCGC TGAACACGCT GGGCTCGAAA  420
GGCGGCAACA ATACCACTTC AACAACAAAT TCCCCGCTGG ACCAGGCGCT GGGTATTAAC  480
TCAACGTCCC AAAACGACGA TTCCACCTCC GGCACAGATT CCACCTCAGA CTCCAGCGAC  540
CCGATGCAGC AGCTGCTGAA GATGTTCAGC GAGATAATGC AAAGCCTGTT TGGTGATGGG  600
CAAGATGGCA CCCAGGGCAG TTCCTCTGGG GGCAAGCAGC CGACCGAAGG CGAGCAGAAC  660
GCCTATAAAA AAGGAGTCAC TGATGCGCTG TCGGGCCTGA TGGGTAATGG TCTGAGCCAG  720
CTCCTTGGCA ACGGGGGACT GGGAGGTGGT CAGGGCGGTA ATGCTGGCAC GGGTCTTGAC  780
GGTTCGTCGC TGGGCGGCAA AGGGCTGCAA AACCTGAGCG GGCCGGTGGA CTACCAGCAG  840
TTAGGTAACG CCGTGGGTAC CGGTATCGGT ATGAAAGCGG GCATTCAGGC GCTGAATGAT  900
ATCGGTACGC ACAGGCACAG TTCAACCCGT TCTTTCGTCA ATAAAGGCGA TCGGGCGATG  960
GCGAAGGAAA TCGGTCAGTT CATGGACCAG TATCCTGAGG TGTTTGGCAA GCCGCAGTAC 1020
CAGAAAGGCC CGGGTCAGGA GGTGAAAACC GATGACAAAT CATGGGCAAA AGCACTGAGC 1080
AAGCCAGATG ACGACGGAAT GACACCAGCC AGTATGGAGC AGTTCAACAA AGCCAAGGGC 1140
ATGATCAAAA GGCCCATGGC GGGTGATACC GGCAACGGCA ACCTGCAGGC ACGCGGTGCC 1200
GGTGGTTCTT CGCTGGGTAT TGATGCCATG ATGGCCGGTG ATGCCATTAA CAATATGGCA 1260
CTTGGCAAGC TGGGCGCGGC TTAAGCTT                                   1288
```

The hypersensitive response elicitor from *Erwinia amylovora* has 2 hypersensitive response eliciting domains. The first domain extends, within SEQ. ID. No. 3, from amino acid 32 to amino acid 74, particularly from amino acid 45 to amino

acid 68. The acidic unit in the first domain extends, within SEQ. ID. No. 3, from amino acid 32 to amino acid 57, particularly from amino acid 45 to amino acid 57. The alpha-helix in the first domain extends, within SEQ. ID. No. 3, from amino acid 57 to amino acid 74, particularly from amino acid 57 to amino acid 68. The second domain extends, within SEQ. ID. No. 3, from amino acid 130 to amino acid 180, particularly from amino acid 145 to amino acid 170. The acidic unit in the second domain extends, within SEQ. ID. No. 3, from amino acid 130 to amino acid 157, particularly from amino acid 145 to amino acid 157. The alpha-helix in the second domain extends, within SEQ. ID. No. 3, from amino acid 157 to amino acid 180, particularly from amino acid 157 to amino acid 170.

Another potentially suitable hypersensitive response elicitor from *Erwinia amylovora* is disclosed in U.S. Patent Application Serial No. 09/120,927, which is hereby incorporated by reference. The protein is encoded by a DNA molecule having a nucleic acid sequence of SEQ. ID. No. 5 as follows:

```
ATGTCAATTC TTACGCTTAA CAACAATACC TCGTCCTCGC CGGGTCTGTT CCAGTCCGGG      60
GGGGACAACG GGCTTGGTGG TCATAATGCA AATTCTGCGT TGGGGCAACA ACCCATCGAT     120
CGGCAAACCA TTGAGCAAAT GGCTCAATTA TTGGCGGAAC TGTTAAAGTC ACTGCTATCG     180
CCACAATCAG GTAATGCGGC AACCGGAGCC GGTGGCAATG ACCAGACTAC AGGAGTTGGT     240
AACGCTGGCG GCCTGAACGG ACGAAAAGGC ACAGCAGGAA CCACTCCGCA GTCTGACAGT     300
CAGAACATGC TGAGTGAGAT GGGCAACAAC GGGCTGGATC AGGCCATCAC GCCCGATGGC     360
CAGGGCGGCG GGCAGATCGG CGATAATCCT TTACTGAAAG CCATGCTGAA GCTTATTGCA     420
CGCATGATGG ACGGCCAAAG CGATCAGTTT GGCCAACCTG GTACGGGCAA CAACAGTGCC     480
TCTTCCGGTA CTTCTTCATC TGGCGGTTCC CCTTTTAACG ATCTATCAGG GGGGAAGGCC     540
CCTTCCGGCA ACTCCCCTTC CGGCAACTAC TCTCCCGTCA GTACCTTCTC ACCCCCATCC     600
ACGCCAACGT CCCCTACCTC ACCGCTTGAT TTCCCTTCTT CTCCCACCAA AGCAGCCGGG     660
GGCAGCACGC CGGTAACCGA TCATCCTGAC CCTGTTGGTA GCGCGGGCAT CGGGGCCGGA     720
AATTCGGTGG CCTTCACCAG CGCCGGCGCT AATCAGACGG TGCTGCATGA CACCATTACC     780
GTGAAAGCGG GTCAGGTGTT TGATGGCAAA GGACAAACCT TCACCGCCGG TTCAGAATTA     840
GGCGATGGCG GCCAGTCTGA AAACCAGAAA CCGCTGTTTA TACTGGAAGA CGGTGCCAGC     900
CTGAAAAACG TCACCATGGG CGACGACGGG GCGGATGGTA TTCATCTTTA CGGTGATGCC     960
AAAATAGACA ATCTGCACGT CACCAACGTG GGTGAGGACG CGATTACCGT TAAGCCAAAC    1020
AGCGCGGGCA AAAAATCCCA CGTTGAAATC ACTAACAGTT CCTTCGAGCA CGCCTCTGAC    1080
```

AAGATCCTGC AGCTGAATGC CGATACTAAC CTGAGCGTTG ACAACGTGAA GGCCAAAGAC 1140

TTTGGTACTT TTGTACGCAC TAACGGCGGT CAACAGGGTA ACTGGGATCT GAATCTGAGC 1200

CATATCAGCG CAGAAGACGG TAAGTTCTCG TTCGTTAAAA GCGATAGCGA GGGGCTAAAC 1260

GTCAATACCA GTGATATCTC ACTGGGTGAT GTTGAAAACC ACTACAAAGT GCCGATGTCC 1320

GCCAACCTGA AGGTGGCTGA ATGA 1344

See GenBank Accession No. U94513. The isolated DNA molecule of the present invention encodes a hypersensitive response elicitor protein or polypeptide having an amino acid sequence of SEQ. ID. No. 6 as follows:

Met Ser Ile Leu Thr Leu Asn Asn Asn Thr Ser Ser Ser Pro Gly Leu
1 5 10 15

Phe Gln Ser Gly Gly Asp Asn Gly Leu Gly Gly His Asn Ala Asn Ser
20 25 30

Ala Leu Gly Gln Gln Pro Ile Asp Arg Gln Thr Ile Glu Gln Met Ala
35 40 45

Gln Leu Leu Ala Glu Leu Leu Lys Ser Leu Leu Ser Pro Gln Ser Gly
50 55 60

Asn Ala Ala Thr Gly Ala Gly Gly Asn Asp Gln Thr Thr Gly Val Gly
65 70 75 80

Asn Ala Gly Gly Leu Asn Gly Arg Lys Gly Thr Ala Gly Thr Thr Pro
85 90 95

Gln Ser Asp Ser Gln Asn Met Leu Ser Glu Met Gly Asn Asn Gly Leu
100 105 110

Asp Gln Ala Ile Thr Pro Asp Gly Gln Gly Gly Gly Gln Ile Gly Asp
115 120 125

Asn Pro Leu Leu Lys Ala Met Leu Lys Leu Ile Ala Arg Met Met Asp
130 135 140

Gly Gln Ser Asp Gln Phe Gly Gln Pro Gly Thr Gly Asn Asn Ser Ala
145 150 155 160

Ser Ser Gly Thr Ser Ser Ser Gly Gly Ser Pro Phe Asn Asp Leu Ser
165 170 175

Gly Gly Lys Ala Pro Ser Gly Asn Ser Pro Ser Gly Asn Tyr Ser Pro
180 185 190

Val Ser Thr Phe Ser Pro Pro Ser Thr Pro Thr Ser Pro Thr Ser Pro
195 200 205

Leu Asp Phe Pro Ser Ser Pro Thr Lys Ala Ala Gly Gly Ser Thr Pro
210 215 220

```
Val Thr Asp His Pro Asp Pro Val Gly Ser Ala Gly Ile Gly Ala Gly
225                 230                 235                 240

Asn Ser Val Ala Phe Thr Ser Ala Gly Ala Asn Gln Thr Val Leu His
                245                 250                 255

Asp Thr Ile Thr Val Lys Ala Gly Gln Val Phe Asp Gly Lys Gly Gln
            260                 265                 270

Thr Phe Thr Ala Gly Ser Glu Leu Gly Asp Gly Gly Gln Ser Glu Asn
        275                 280                 285

Gln Lys Pro Leu Phe Ile Leu Glu Asp Gly Ala Ser Leu Lys Asn Val
    290                 295                 300

Thr Met Gly Asp Asp Gly Ala Asp Gly Ile His Leu Tyr Gly Asp Ala
305                 310                 315                 320

Lys Ile Asp Asn Leu His Val Thr Asn Val Gly Glu Asp Ala Ile Thr
                325                 330                 335

Val Lys Pro Asn Ser Ala Gly Lys Lys Ser His Val Glu Ile Thr Asn
            340                 345                 350

Ser Ser Phe Glu His Ala Ser Asp Lys Ile Leu Gln Leu Asn Ala Asp
        355                 360                 365

Thr Asn Leu Ser Val Asp Asn Val Lys Ala Lys Asp Phe Gly Thr Phe
    370                 375                 380

Val Arg Thr Asn Gly Gly Gln Gln Gly Asn Trp Asp Leu Asn Leu Ser
385                 390                 395                 400

His Ile Ser Ala Glu Asp Gly Lys Phe Ser Phe Val Lys Ser Asp Ser
                405                 410                 415

Glu Gly Leu Asn Val Asn Thr Ser Asp Ile Ser Leu Gly Asp Val Glu
            420                 425                 430

Asn His Tyr Lys Val Pro Met Ser Ala Asn Leu Lys Val Ala Glu
        435                 440                 445
```

This protein or polypeptide is acidic, rich in glycine and serine, and lacks cysteine. It is also heat stable, protease sensitive, and suppressed by inhibitors of plant metabolism. The protein or polypeptide of the present invention has a predicted molecular size of ca. 4.5 kDa.

This hypersensitive response elicitor from *Erwinia amylovora* has 2 hypersensitive response eliciting domains. The first domain extends, within SEQ. ID. No. 6, from amino acid 5 to amino acid 64, particularly from amino acid 31 to amino acid 57. The acidic unit in the first domain extends, within SEQ. ID. No. 6, from amino acid 5 to amino acid 45, particularly from amino acid 31 to amino acid 45. The

alpha-helix in the first domain extends, within SEQ. ID. No. 6, from amino acid 45 to amino acid 64, particularly from amino acid 45 to amino acid 64. The second domain extends, within SEQ. ID. No. 6, from amino acid 103 to amino acid 146, particularly from amino acid 116 to amino acid 140. The acidic unit in the second domain extends, within SEQ. ID. No. 6, from amino acid 103 to amino acid 131, particularly from amino acid 116 to amino acid 131. The alpha-helix in the second domain extends, within SEQ. ID. No. 6, from amino acid 131 to amino acid 146, particularly from amino acid 131 to amino acid 140.

Another potentially suitable hypersensitive response elicitor from *Erwinia amylovora* is disclosed in U.S. Patent Application Serial No. 09/120,663, which is hereby incorporated by reference. The protein is encoded by a DNA molecule having a nucleic acid sequence of SEQ. ID. No. 7 as follows:

```
ATGGAATTAA AATCACTGGG AACTGAACAC AAGGCGGCAG TACACACAGC GGCGCACAAC     60
CCTGTGGGGC ATGGTGTTGC CTTACAGCAG GGCAGCAGCA GCAGCAGCCC GCAAAATGCC    120
GCTGCATCAT TGGCGGCAGA AGGCAAAAAT CGTGGGAAAA TGCCGAGAAT TCACCAGCCA    180
TCTACTGCGG CTGATGGTAT CAGCGCTGCT CACCAGCAAA AGAAATCCTT CAGTCTCAGG    240
GGCTGTTTGG GGACGAAAAA ATTTTCCAGA TCGGCACCGC AGGGCCAGCC AGGTACCACC    300
CACAGCAAAG GGGCAACATT GCGCGATCTG CTGGCGCGGG ACGACGGCGA AACGCAGCAT    360
GAGGCGGCCG CGCCAGATGC GGCGCGTTTG ACCCGTTCGG GCGGCGTCAA ACGCCGCAAT    420
ATGGACGACA TGGCCGGGCG GCCAATGGTG AAAGGTGGCA GCGGCGAAGA TAAGGTACCA    480
ACGCAGCAAA AACGGCATCA GCTGAACAAT TTTGGCCAGA TGCGCCAAAC GATGTTGAGC    540
AAAATGGCTC ACCCGGCTTC AGCCAACGCC GGCGATCGCC TGCAGCATTC ACCGCCGCAC    600
ATCCCGGGTA GCCACCACGA AATCAAGGAA GAACCGGTTG GCTCCACCAG CAAGGCAACA    660
ACGGCCCACG CAGACAGAGT GGAAATCGCT CAGGAAGATG ACGACAGCGA ATTCCAGCAA    720
CTGCATCAAC AGCGGCTGGC GCGCGAACGG GAAAATCCAC CGCAGCCGCC CAAACTCGGC    780
GTTGCCACAC CGATTAGCGC CAGGTTTCAG CCCAAACTGA CTGCGGTTGC GGAAAGCGTC    840
CTTGAGGGGA CAGATACCAC GCAGTCACCC CTTAAGCCGC AATCAATGCT GAAAGGAAGT    900
GGAGCCGGGG TAACGCCGCT GGCGGTAACG CTGGATAAAG GCAAGTTGCA GCTGGCACCG    960
GATAATCCAC CCGCGCTCAA TACGTTGTTG AAGCAGACAT TGGGTAAAGA CACCCAGCAC   1020
TATCTGGCGC ACCATGCCAG CAGCGACGGT AGCCAGCATC TGCTGCTGGA CAACAAAGGC   1080
CACCTGTTTG ATATCAAAAG CACCGCCACC AGCTATAGCG TGCTGCACAA CAGCCACCCC   1140
GGTGAGATAA AGGGCAAGCT GGCGCAGGCG GGTACTGGCT CCGTCAGCGT AGACGGTAAA   1200
```

AGCGGCAAGA TCTCGCTGGG GAGCGGTACG CAAAGTCACA ACAAAACAAT GCTAAGCCAA 1260
CCGGGGGAAG CGCACCGTTC CTTATTAACC GGCATTTGGC AGCATCCTGC TGGCGCAGCG 1320
CGGCCGCAGG GCGAGTCAAT CCGCCTGCAT GACGACAAAA TTCATATCCT GCATCCGGAG 1380
CTGGGCGTAT GGCAATCTGC GGATAAAGAT ACCCACAGCC AGCTGTCTCG CCAGGCAGAC 1440
GGTAAGCTCT ATGCGCTGAA AGACAACCGT ACCCTGCAAA ACCTCTCCGA TAATAAATCC 1500
TCAGAAAAGC TGGTCGATAA AATCAAATCG TATTCCGTTG ATCAGCGGGG GCAGGTGGCG 1560
ATCCTGACGG ATACTCCCGG CCGCCATAAG ATGAGTATTA TGCCCTCGCT GGATGCTTCC 1620
CCGGAGAGCC ATATTTCCCT CAGCCTGCAT TTTGCCGATG CCCACCAGGG GTTATTGCAC 1680
GGGAAGTCGG AGCTTGAGGC ACAATCTGTC GCGATCAGCC ATGGGCGACT GGTTGTGGCC 1740
GATAGCGAAG GCAAGCTGTT TAGCGCCGCC ATTCCGAAGC AAGGGGATGG AAACGAACTG 1800
AAAATGAAAG CCATGCCTCA GCATGCGCTC GATGAACATT TTGGTCATGA CCACCAGATT 1860
TCTGGATTTT TCCATGACGA CCACGGCCAG CTTAATGCGC TGGTGAAAAA TAACTTCAGG 1920
CAGCAGCATG CCTGCCCGTT GGGTAACGAT CATCAGTTTC ACCCCGGCTG GAACCTGACT 1980
GATGCGCTGG TTATCGACAA TCAGCTGGGG CTGCATCATA CCAATCCTGA ACCGCATGAG 2040
ATTCTTGATA TGGGGCATTT AGGCAGCCTG GCGTTACAGG AGGGCAAGCT TCACTATTTT 2100
GACCAGCTGA CCAAAGGGTG GACTGGCGCG GAGTCAGATT GTAAGCAGCT GAAAAAAGGC 2160
CTGGATGGAG CAGCTTATCT ACTGAAAGAC GGTGAAGTGA AACGCCTGAA TATTAATCAG 2220
AGCACCTCCT CTATCAAGCA CGGAACGGAA AACGTTTTTT CGCTGCCGCA TGTGCGCAAT 2280
AAACCGGAGC CGGGAGATGC CCTGCAAGGG CTGAATAAAG ACGATAAGGC CCAGGCCATG 2340
GCGGTGATTG GGGTAAATAA ATACCTGGCG CTGACGGAAA AAGGGGACAT TCGCTCCTTC 2400
CAGATAAAAC CCGGCACCCA GCAGTTGGAG CGGCCGGCAC AAACTCTCAG CCGCGAAGGT 2460
ATCAGCGGCG AACTGAAAGA CATTCATGTC GACCACAAGC AGAACCTGTA TGCCTTGACC 2520
CACGAGGGAG AGGTGTTTCA TCAGCCGCGT GAAGCCTGGC AGAATGGTGC CGAAAGCAGC 2580
AGCTGGCACA AACTGGCGTT GCCACAGAGT GAAAGTAAGC TAAAAAGTCT GGACATGAGC 2640
CATGAGCACA AACCGATTGC CACCTTTGAA GACGGTAGCC AGCATCAGCT GAAGGCTGGC 2700
GGCTGGCACG CCTATGCGGC ACCTGAACGC GGGCCGCTGG CGGTGGGTAC CAGCGGTTCA 2760
CAAACCGTCT TTAACCGACT AATGCAGGGG GTGAAAGGCA AGGTGATCCC AGGCAGCGGG 2820
TTGACGGTTA AGCTCTCGGC TCAGACGGGG GGAATGACCG GCGCCGAAGG GCGCAAGGTC 2880
AGCAGTAAAT TTTCCGAAAG GATCCGCGCC TATGCGTTCA ACCCAACAAT GTCCACGCCG 2940
CGACCGATTA AAAATGCTGC TTATGCCACA CAGCACGGCT GGCAGGGGCG TGAGGGGTTG 3000
AAGCCGTTGT ACGAGATGCA GGGAGCGCTG ATTAAACAAC TGGATGCGCA TAACGTTCGT 3060
CATAACGCGC CACAGCCAGA TTTGCAGAGC AAACTGGAAA CTCTGGATTT AGGCGAACAT 3120
GGCGCAGAAT TGCTTAACGA CATGAAGCGC TTCCGCGACG AACTGGAGCA GAGTGCAACC 3180

CGTTCGGTGA CCGTTTTAGG TCAACATCAG GGAGTGCTAA AAAGCAACGG TGAAATCAAT 3240
AGCGAATTTA AGCCATCGCC CGGCAAGGCG TTGGTCCAGA GCTTTAACGT CAATCGCTCT 3300
GGTCAGGATC TAAGCAAGTC ACTGCAACAG GCAGTACATG CCACGCCGCC ATCCGCAGAG 3360
AGTAAACTGC AATCCATGCT GGGGCACTTT GTCAGTGCCG GGGTGGATAT GAGTCATCAG 3420
AAGGGCGAGA TCCCGCTGGG CCGCCAGCGC GATCCGAATG ATAAAACCGC ACTGACCAAA 3480
TCGCGTTTAA TTTTAGATAC CGTGACCATC GGTGAACTGC ATGAACTGGC CGATAAGGCG 3540
AAACTGGTAT CTGACCATAA ACCCGATGCC GATCAGATAA AACAGCTGCG CCAGCAGTTC 3600
GATACGCTGC GTGAAAAGCG GTATGAGAGC AATCCGGTGA AGCATTACAC CGATATGGGC 3660
TTCACCCATA ATAAGGCGCT GGAAGCAAAC TATGATGCGG TCAAAGCCTT TATCAATGCC 3720
TTTAAGAAAG AGCACCACGG CGTCAATCTG ACCACGCGTA CCGTACTGGA ATCACAGGGC 3780
AGTGCGGAGC TGGCGAAGAA GCTCAAGAAT ACGCTGTTGT CCCTGGACAG TGGTGAAAGT 3840
ATGAGCTTCA GCCGGTCATA TGGCGGGGGC GTCAGCACTG TCTTTGTGCC TACCCTTAGC 3900
AAGAAGGTGC CAGTTCCGGT GATCCCCGGA GCCGGCATCA CGCTGGATCG CGCCTATAAC 3960
CTGAGCTTCA GTCGTACCAG CGGCGGATTG AACGTCAGTT TTGGCCGCGA CGGCGGGGTG 4020
AGTGGTAACA TCATGGTCGC TACCGGCCAT GATGTGATGC CCTATATGAC CGGTAAGAAA 4080
ACCAGTGCAG GTAACGCCAG TGACTGGTTG AGCGCAAAAC ATAAAATCAG CCCGGACTTG 4140
CGTATCGGCG CTGCTGTGAG TGGCACCCTG CAAGGAACGC TACAAAACAG CCTGAAGTTT 4200
AAGCTGACAG AGGATGAGCT GCCTGGCTTT ATCCATGGCT TGACGCATGG CACGTTGACC 4260
CCGGCAGAAC TGTTGCAAAA GGGGATCGAA CATCAGATGA AGCAGGGCAG CAAACTGACG 4320
TTTAGCGTCG ATACCTCGGC AAATCTGGAT CTGCGTGCCG GTATCAATCT GAACGAAGAC 4380
GGCAGTAAAC CAAATGGTGT CACTGCCCGT GTTTCTGCCG GGCTAAGTGC ATCGGCAAAC 4440
CTGGCCGCCG GCTCGCGTGA ACGCAGCACC ACCTCTGGCC AGTTTGGCAG CACGACTTCG 4500
GCCAGCAATA ACCGCCCAAC CTTCCTCAAC GGGGTCGGCG CGGGTGCTAA CCTGACGGCT 4560
GCTTTAGGGG TTGCCCATTC ATCTACGCAT GAAGGGAAAC CGGTCGGGAT CTTCCCGGCA 4620
TTTACCTCGA CCAATGTTTC GGCAGCGCTG GCGCTGGATA ACCGTACCTC ACAGAGTATC 4680
AGCCTGGAAT TGAAGCGCGC GGAGCCGGTG ACCAGCAACG ATATCAGCGA GTTGACCTCC 4740
ACGCTGGGAA AACACTTTAA GGATAGCGCC ACAACGAAGA TGCTTGCCGC TCTCAAAGAG 4800
TTAGATGACG CTAAGCCCGC TGAACAACTG CATATTTTAC AGCAGCATTT CAGTGCAAAA 4860
GATGTCGTCG GTGATGAACG CTACGAGGCG GTGCGCAACC TGAAAAAACT GGTGATACGT 4920
CAACAGGCTG CGGACAGCCA CAGCATGGAA TTAGGATCTG CCAGTCACAG CACGACCTAC 4980
AATAATCTGT CGAGAATAAA TAATGACGGC ATTGTCGAGC TGCTACACAA ACATTTCGAT 5040
GCGGCATTAC CAGCAAGCAG TGCCAAACGT CTTGGTGAAA TGATGAATAA CGATCCGGCA 5100

```
CTGAAAGATA TTATTAAGCA GCTGCAAAGT ACGCCGTTCA GCAGCGCCAG CGTGTCGATG   5160
GAGCTGAAAG ATGGTCTGCG TGAGCAGACG GAAAAAGCAA TACTGGACGG TAAGGTCGGT   5220
CGTGAAGAAG TGGGAGTACT TTTCCAGGAT CGTAACAACT TGCGTGTTAA ATCGGTCAGC   5280
GTCAGTCAGT CCGTCAGCAA AAGCGAAGGC TTCAATACCC CAGCGCTGTT ACTGGGGACG   5340
AGCAACAGCG CTGCTATGAG CATGGAGCGC AACATCGGAA CCATTAATTT TAAATACGGC   5400
CAGGATCAGA ACACCCCACG GCGATTTACC CTGGAGGGTG GAATAGCTCA GGCTAATCCG   5460
CAGGTCGCAT CTGCGCTTAC TGATTTGAAG AAGGAAGGGC TGGAAATGAA GAGCTAA      5517
```

This DNA molecule is known as the dspE gene for *Erwinia amylovora*. This isolated DNA molecule of the present invention encodes a protein or polypeptide which elicits a plant pathogen's hypersensitive response having an amino acid sequence of SEQ. ID. No. 8 as follows:

```
Met Glu Leu Lys Ser Leu Gly Thr Glu His Lys Ala Ala Val His Thr
1               5                   10                  15

Ala Ala His Asn Pro Val Gly His Gly Val Ala Leu Gln Gln Gly Ser
            20                  25                  30

Ser Ser Ser Ser Pro Gln Asn Ala Ala Ala Ser Leu Ala Ala Glu Gly
        35                  40                  45

Lys Asn Arg Gly Lys Met Pro Arg Ile His Gln Pro Ser Thr Ala Ala
    50                  55                  60

Asp Gly Ile Ser Ala Ala His Gln Gln Lys Lys Ser Phe Ser Leu Arg
65                  70                  75                  80

Gly Cys Leu Gly Thr Lys Lys Phe Ser Arg Ser Ala Pro Gln Gly Gln
                85                  90                  95

Pro Gly Thr Thr His Ser Lys Gly Ala Thr Leu Arg Asp Leu Leu Ala
            100                 105                 110

Arg Asp Asp Gly Glu Thr Gln His Glu Ala Ala Ala Pro Asp Ala Ala
        115                 120                 125

Arg Leu Thr Arg Ser Gly Gly Val Lys Arg Arg Asn Met Asp Asp Met
    130                 135                 140

Ala Gly Arg Pro Met Val Lys Gly Gly Ser Gly Glu Asp Lys Val Pro
145                 150                 155                 160

Thr Gln Gln Lys Arg His Gln Leu Asn Asn Phe Gly Gln Met Arg Gln
                165                 170                 175

Thr Met Leu Ser Lys Met Ala His Pro Ala Ser Ala Asn Ala Gly Asp
            180                 185                 190

Arg Leu Gln His Ser Pro Pro His Ile Pro Gly Ser His His Glu Ile
        195                 200                 205
```

Lys Glu Glu Pro Val Gly Ser Thr Ser Lys Ala Thr Thr Ala His Ala
210 215 220

Asp Arg Val Glu Ile Ala Gln Glu Asp Asp Asp Ser Glu Phe Gln Gln
225 230 235 240

Leu His Gln Gln Arg Leu Ala Arg Glu Arg Glu Asn Pro Pro Gln Pro
245 250 255

Pro Lys Leu Gly Val Ala Thr Pro Ile Ser Ala Arg Phe Gln Pro Lys
260 265 270

Leu Thr Ala Val Ala Glu Ser Val Leu Glu Gly Thr Asp Thr Thr Gln
275 280 285

Ser Pro Leu Lys Pro Gln Ser Met Leu Lys Gly Ser Gly Ala Gly Val
290 295 300

Thr Pro Leu Ala Val Thr Leu Asp Lys Gly Lys Leu Gln Leu Ala Pro
305 310 315 320

Asp Asn Pro Pro Ala Leu Asn Thr Leu Leu Lys Gln Thr Leu Gly Lys
325 330 335

Asp Thr Gln His Tyr Leu Ala His His Ala Ser Ser Asp Gly Ser Gln
340 345 350

His Leu Leu Leu Asp Asn Lys Gly His Leu Phe Asp Ile Lys Ser Thr
355 360 365

Ala Thr Ser Tyr Ser Val Leu His Asn Ser His Pro Gly Glu Ile Lys
370 375 380

Gly Lys Leu Ala Gln Ala Gly Thr Gly Ser Val Ser Val Asp Gly Lys
385 390 395 400

Ser Gly Lys Ile Ser Leu Gly Ser Gly Thr Gln Ser His Asn Lys Thr
405 410 415

Met Leu Ser Gln Pro Gly Glu Ala His Arg Ser Leu Leu Thr Gly Ile
420 425 430

Trp Gln His Pro Ala Gly Ala Ala Arg Pro Gln Gly Glu Ser Ile Arg
435 440 445

Leu His Asp Asp Lys Ile His Ile Leu His Pro Glu Leu Gly Val Trp
450 455 460

Gln Ser Ala Asp Lys Asp Thr His Ser Gln Leu Ser Arg Gln Ala Asp
465 470 475 480

Gly Lys Leu Tyr Ala Leu Lys Asp Asn Arg Thr Leu Gln Asn Leu Ser
485 490 495

Asp Asn Lys Ser Ser Glu Lys Leu Val Asp Lys Ile Lys Ser Tyr Ser
500 505 510

Val Asp Gln Arg Gly Gln Val Ala Ile Leu Thr Asp Thr Pro Gly Arg
515 520 525

His Lys Met Ser Ile Met Pro Ser Leu Asp Ala Ser Pro Glu Ser His
530 535 540

Ile Ser Leu Ser Leu His Phe Ala Asp Ala His Gln Gly Leu Leu His
545 550 555 560

Gly Lys Ser Glu Leu Glu Ala Gln Ser Val Ala Ile Ser His Gly Arg
565 570 575

Leu Val Val Ala Asp Ser Glu Gly Lys Leu Phe Ser Ala Ala Ile Pro
580 585 590

Lys Gln Gly Asp Gly Asn Glu Leu Lys Met Lys Ala Met Pro Gln His
595 600 605

Ala Leu Asp Glu His Phe Gly His Asp His Gln Ile Ser Gly Phe Phe
610 615 620

His Asp Asp His Gly Gln Leu Asn Ala Leu Val Lys Asn Asn Phe Arg
625 630 635 640

Gln Gln His Ala Cys Pro Leu Gly Asn Asp His Gln Phe His Pro Gly
645 650 655

Trp Asn Leu Thr Asp Ala Leu Val Ile Asp Asn Gln Leu Gly Leu His
660 665 670

His Thr Asn Pro Glu Pro His Glu Ile Leu Asp Met Gly His Leu Gly
675 680 685

Ser Leu Ala Leu Gln Glu Gly Lys Leu His Tyr Phe Asp Gln Leu Thr
690 695 700

Lys Gly Trp Thr Gly Ala Glu Ser Asp Cys Lys Gln Leu Lys Lys Gly
705 710 715 720

Leu Asp Gly Ala Ala Tyr Leu Leu Lys Asp Gly Glu Val Lys Arg Leu
725 730 735

Asn Ile Asn Gln Ser Thr Ser Ser Ile Lys His Gly Thr Glu Asn Val
740 745 750

Phe Ser Leu Pro His Val Arg Asn Lys Pro Glu Pro Gly Asp Ala Leu
755 760 765

Gln Gly Leu Asn Lys Asp Asp Lys Ala Gln Ala Met Ala Val Ile Gly
770 775 780

Val Asn Lys Tyr Leu Ala Leu Thr Glu Lys Gly Asp Ile Arg Ser Phe
785 790 795 800

Gln Ile Lys Pro Gly Thr Gln Gln Leu Glu Arg Pro Ala Gln Thr Leu
805 810 815

Ser Arg Glu Gly Ile Ser Gly Glu Leu Lys Asp Ile His Val Asp His
820 825 830

Lys Gln Asn Leu Tyr Ala Leu Thr His Glu Gly Glu Val Phe His Gln
835 840 845

Pro Arg Glu Ala Trp Gln Asn Gly Ala Glu Ser Ser Ser Trp His Lys
850 855 860

Leu Ala Leu Pro Gln Ser Glu Ser Lys Leu Lys Ser Leu Asp Met Ser
865 870 875 880

His Glu His Lys Pro Ile Ala Thr Phe Glu Asp Gly Ser Gln His Gln
885 890 895

Leu Lys Ala Gly Gly Trp His Ala Tyr Ala Ala Pro Glu Arg Gly Pro
900 905 910

Leu Ala Val Gly Thr Ser Gly Ser Gln Thr Val Phe Asn Arg Leu Met
915 920 925

Gln Gly Val Lys Gly Lys Val Ile Pro Gly Ser Gly Leu Thr Val Lys
930 935 940

Leu Ser Ala Gln Thr Gly Gly Met Thr Gly Ala Glu Gly Arg Lys Val
945 950 955 960

Ser Ser Lys Phe Ser Glu Arg Ile Arg Ala Tyr Ala Phe Asn Pro Thr
965 970 975

Met Ser Thr Pro Arg Pro Ile Lys Asn Ala Ala Tyr Ala Thr Gln His
980 985 990

Gly Trp Gln Gly Arg Glu Gly Leu Lys Pro Leu Tyr Glu Met Gln Gly
995 1000 1005

Ala Leu Ile Lys Gln Leu Asp Ala His Asn Val Arg His Asn Ala Pro
1010 1015 1020

Gln Pro Asp Leu Gln Ser Lys Leu Glu Thr Leu Asp Leu Gly Glu His
1025 1030 1035 1040

Gly Ala Glu Leu Leu Asn Asp Met Lys Arg Phe Arg Asp Glu Leu Glu
1045 1050 1055

Gln Ser Ala Thr Arg Ser Val Thr Val Leu Gly Gln His Gln Gly Val
1060 1065 1070

Leu Lys Ser Asn Gly Glu Ile Asn Ser Glu Phe Lys Pro Ser Pro Gly
1075 1080 1085

Lys Ala Leu Val Gln Ser Phe Asn Val Asn Arg Ser Gly Gln Asp Leu
1090 1095 1100

Ser Lys Ser Leu Gln Gln Ala Val His Ala Thr Pro Pro Ser Ala Glu
1105 1110 1115 1120

Ser Lys Leu Gln Ser Met Leu Gly His Phe Val Ser Ala Gly Val Asp
1125 1130 1135

Met Ser His Gln Lys Gly Glu Ile Pro Leu Gly Arg Gln Arg Asp Pro
1140 1145 1150

Asn Asp Lys Thr Ala Leu Thr Lys Ser Arg Leu Ile Leu Asp Thr Val
1155 1160 1165

Thr Ile Gly Glu Leu His Glu Leu Ala Asp Lys Ala Lys Leu Val Ser
1170 1175 1180

Asp His Lys Pro Asp Ala Asp Gln Ile Lys Gln Leu Arg Gln Gln Phe
1185 1190 1195 1200

Asp Thr Leu Arg Glu Lys Arg Tyr Glu Ser Asn Pro Val Lys His Tyr
1205 1210 1215

Thr Asp Met Gly Phe Thr His Asn Lys Ala Leu Glu Ala Asn Tyr Asp
1220 1225 1230

Ala Val Lys Ala Ph Ile Asn Ala Phe Lys Lys Glu His His Gly Val
1235 1240 1245

```
Asn Leu Thr Thr Arg Thr Val Leu Glu Ser Gln Gly Ser Ala Glu Leu
    1250                1255                1260

Ala Lys Lys Leu Lys Asn Thr Leu Leu Ser Leu Asp Ser Gly Glu Ser
1265                1270                1275                1280

Met Ser Phe Ser Arg Ser Tyr Gly Gly Gly Val Ser Thr Val Phe Val
                1285                1290                1295

Pro Thr Leu Ser Lys Lys Val Pro Val Pro Val Ile Pro Gly Ala Gly
            1300                1305                1310

Ile Thr Leu Asp Arg Ala Tyr Asn Leu Ser Phe Ser Arg Thr Ser Gly
        1315                1320                1325

Gly Leu Asn Val Ser Phe Gly Arg Asp Gly Gly Val Ser Gly Asn Ile
    1330                1335                1340

Met Val Ala Thr Gly His Asp Val Met Pro Tyr Met Thr Gly Lys Lys
1345                1350                1355                1360

Thr Ser Ala Gly Asn Ala Ser Asp Trp Leu Ser Ala Lys His Lys Ile
                1365                1370                1375

Ser Pro Asp Leu Arg Ile Gly Ala Ala Val Ser Gly Thr Leu Gln Gly
            1380                1385                1390

Thr Leu Gln Asn Ser Leu Lys Phe Lys Leu Thr Glu Asp Glu Leu Pro
        1395                1400                1405

Gly Phe Ile His Gly Leu Thr His Gly Thr Leu Thr Pro Ala Glu Leu
    1410                1415                1420

Leu Gln Lys Gly Ile Glu His Gln Met Lys Gln Gly Ser Lys Leu Thr
1425                1430                1435                1440

Phe Ser Val Asp Thr Ser Ala Asn Leu Asp Leu Arg Ala Gly Ile Asn
                1445                1450                1455

Leu Asn Glu Asp Gly Ser Lys Pro Asn Gly Val Thr Ala Arg Val Ser
            1460                1465                1470

Ala Gly Leu Ser Ala Ser Ala Asn Leu Ala Ala Gly Ser Arg Glu Arg
        1475                1480                1485

Ser Thr Thr Ser Gly Gln Phe Gly Ser Thr Thr Ser Ala Ser Asn Asn
    1490                1495                1500

Arg Pro Thr Phe Leu Asn Gly Val Gly Ala Gly Ala Asn Leu Thr Ala
1505                1510                1515                1520

Ala Leu Gly Val Ala His Ser Ser Thr His Glu Gly Lys Pro Val Gly
                1525                1530                1535

Ile Phe Pro Ala Phe Thr Ser Thr Asn Val Ser Ala Ala Leu Ala Leu
            1540                1545                1550

Asp Asn Arg Thr Ser Gln Ser Ile Ser Leu Glu Leu Lys Arg Ala Glu
        1555                1560                1565

Pro Val Thr Ser Asn Asp Ile Ser Glu Leu Thr Ser Thr Leu Gly Lys
    1570                1575                1580
```

His Phe Lys Asp Ser Ala Thr Thr Lys Met Leu Ala Ala Leu Lys Glu
1585 1590 1595 1600

Leu Asp Asp Ala Lys Pro Ala Glu Gln Leu His Ile Leu Gln Gln His
1605 1610 1615

Phe Ser Ala Lys Asp Val Val Gly Asp Glu Arg Tyr Glu Ala Val Arg
1620 1625 1630

Asn Leu Lys Lys Leu Val Ile Arg Gln Gln Ala Ala Asp Ser His Ser
1635 1640 1645

Met Glu Leu Gly Ser Ala Ser His Ser Thr Thr Tyr Asn Asn Leu Ser
1650 1655 1660

Arg Ile Asn Asn Asp Gly Ile Val Glu Leu Leu His Lys His Phe Asp
1665 1670 1675 1680

Ala Ala Leu Pro Ala Ser Ser Ala Lys Arg Leu Gly Glu Met Met Asn
1685 1690 1695

Asn Asp Pro Ala Leu Lys Asp Ile Ile Lys Gln Leu Gln Ser Thr Pro
1700 1705 1710

Phe Ser Ser Ala Ser Val Ser Met Glu Leu Lys Asp Gly Leu Arg Glu
1715 1720 1725

Gln Thr Glu Lys Ala Ile Leu Asp Gly Lys Val Gly Arg Glu Glu Val
1730 1735 1740

Gly Val Leu Phe Gln Asp Arg Asn Asn Leu Arg Val Lys Ser Val Ser
1745 1750 1755 1760

Val Ser Gln Ser Val Ser Lys Ser Glu Gly Phe Asn Thr Pro Ala Leu
1765 1770 1775

Leu Leu Gly Thr Ser Asn Ser Ala Ala Met Ser Met Glu Arg Asn Ile
1780 1785 1790

Gly Thr Ile Asn Phe Lys Tyr Gly Gln Asp Gln Asn Thr Pro Arg Arg
1795 1800 1805

Phe Thr Leu Glu Gly Gly Ile Ala Gln Ala Asn Pro Gln Val Ala Ser
1810 1815 1820

Ala Leu Thr Asp Leu Lys Lys Glu Gly Leu Glu Met Lys Ser
1825 1830 1835

This protein or polypeptide is about 198 kDa and has a pI of 8.98.

The present invention relates to an isolated DNA molecule having a nucleotide sequence of SEQ. ID. No. 9 as follows:

ATGACATCGT CACAGCAGCG GGTTGAAAGG TTTTTACAGT ATTTCTCCGC CGGGTGTAAA 60

ACGCCCATAC ATCTGAAAGA CGGGGTGTGC GCCCTGTATA ACGAACAAGA TGAGGAGGCG 120

GCGGTGCTGG AAGTACCGCA ACACAGCGAC AGCCTGTTAC TACACTGCCG AATCATTGAG 180

GCTGACCCAC AAACTTCAAT AACCCTGTAT TCGATGCTAT TACAGCTGAA TTTTGAAATG 240

```
GCGGCCATGC GCGGCTGTTG GCTGGCGCTG GATGAACTGC ACAACGTGCG TTTATGTTTT      300

CAGCAGTCGC TGGAGCATCT GGATGAAGCA AGTTTTAGCG ATATCGTTAG CGGCTTCATC      360

GAACATGCGG CAGAAGTGCG TGAGTATATA GCGCAATTAG ACGAGAGTAG CGCGGCATAA      420
```

This is known as the dspF gene. This isolated DNA molecule of the present invention encodes a hypersensitive response elicitor protein or polypeptide having an amino acid sequence of SEQ. ID. No. 10 as follows:

```
Met Thr Ser Ser Gln Gln Arg Val Glu Arg Phe Leu Gln Tyr Phe Ser
1               5                   10                  15

Ala Gly Cys Lys Thr Pro Ile His Leu Lys Asp Gly Val Cys Ala Leu
            20                  25                  30

Tyr Asn Glu Gln Asp Glu Glu Ala Ala Val Leu Glu Val Pro Gln His
        35                  40                  45

Ser Asp Ser Leu Leu Leu His Cys Arg Ile Ile Glu Ala Asp Pro Gln
    50                  55                  60

Thr Ser Ile Thr Leu Tyr Ser Met Leu Leu Gln Leu Asn Phe Glu Met
65                  70                  75                  80

Ala Ala Met Arg Gly Cys Trp Leu Ala Leu Asp Glu Leu His Asn Val
                85                  90                  95

Arg Leu Cys Phe Gln Gln Ser Leu Glu His Leu Asp Glu Ala Ser Phe
            100                 105                 110

Ser Asp Ile Val Ser Gly Phe Ile Glu His Ala Ala Glu Val Arg Glu
        115                 120                 125

Tyr Ile Ala Gln Leu Asp Glu Ser Ser Ala Ala
    130                 135
```

This protein or polypeptide is about 16 kDa and has a pI of 4.45.

The hypersensitive response elicitor polypeptide or protein derived from *Pseudomonas syringae* has an amino acid sequence corresponding to SEQ. ID. No. 11 as follows:

```
Met Gln Ser Leu Ser Leu Asn Ser Ser Ser Leu Gln Thr Pro Ala Met
1               5                   10                  15

Ala Leu Val Leu Val Arg Pro Glu Ala Glu Thr Thr Gly Ser Thr Ser
            20                  25                  30

Ser Lys Ala Leu Gln Glu Val Val Val Lys Leu Ala Glu Glu Leu Met
        35                  40                  45

Arg Asn Gly Gln Leu Asp Asp Ser Ser Pro Leu Gly Lys Leu Leu Ala
    50                  55                  60
```

Lys Ser Met Ala Ala Asp Gly Lys Ala Gly Gly Gly Ile Glu Asp Val
65 70 75 80

Ile Ala Ala Leu Asp Lys Leu Ile His Glu Lys Leu Gly Asp Asn Phe
85 90 95

Gly Ala Ser Ala Asp Ser Ala Ser Gly Thr Gly Gln Gln Asp Leu Met
100 105 110

Thr Gln Val Leu Asn Gly Leu Ala Lys Ser Met Leu Asp Asp Leu Leu
115 120 125

Thr Lys Gln Asp Gly Gly Thr Ser Phe Ser Glu Asp Asp Met Pro Met
130 135 140

Leu Asn Lys Ile Ala Gln Phe Met Asp Asp Asn Pro Ala Gln Phe Pro
145 150 155 160

Lys Pro Asp Ser Gly Ser Trp Val Asn Glu Leu Lys Glu Asp Asn Phe
165 170 175

Leu Asp Gly Asp Glu Thr Ala Ala Phe Arg Ser Ala Leu Asp Ile Ile
180 185 190

Gly Gln Gln Leu Gly Asn Gln Gln Ser Asp Ala Gly Ser Leu Ala Gly
195 200 205

Thr Gly Gly Gly Leu Gly Thr Pro Ser Ser Phe Ser Asn Asn Ser Ser
210 215 220

Val Met Gly Asp Pro Leu Ile Asp Ala Asn Thr Gly Pro Gly Asp Ser
225 230 235 240

Gly Asn Thr Arg Gly Glu Ala Gly Gln Leu Ile Gly Glu Leu Ile Asp
245 250 255

Arg Gly Leu Gln Ser Val Leu Ala Gly Gly Gly Leu Gly Thr Pro Val
260 265 270

Asn Thr Pro Gln Thr Gly Thr Ser Ala Asn Gly Gly Gln Ser Ala Gln
275 280 285

Asp Leu Asp Gln Leu Leu Gly Gly Leu Leu Leu Lys Gly Leu Glu Ala
290 295 300

Thr Leu Lys Asp Ala Gly Gln Thr Gly Thr Asp Val Gln Ser Ser Ala
305 310 315 320

Ala Gln Ile Ala Thr Leu Leu Val Ser Thr Leu Leu Gln Gly Thr Arg
325 330 335

Asn Gln Ala Ala Ala
340

This hypersensitive response elicitor polypeptide or protein has a molecular weight of 34-35 kDa. It is rich in glycine (about 13.5%) and lacks cysteine and tyrosine. Further information about the hypersensitive response elicitor derived from *Pseudomonas syringae* is found in He, S. Y., H. C. Huang, and A. Collmer, "*Pseudomonas syringae* pv. *syringae* Harpin$_{Pss}$: a Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," Cell 73:1255-1266 (1993), which is hereby incorporated by reference. The DNA molecule encoding the hypersensitive response elicitor from *Pseudomonas syringae* has a nucleotide sequence corresponding to SEQ. ID. No. 12 as follows:

```
ATGCAGAGTC TCAGTCTTAA CAGCAGCTCG CTGCAAACCC CGGCAATGGC CCTTGTCCTG     60
GTACGTCCTG AAGCCGAGAC GACTGGCAGT ACGTCGAGCA AGGCGCTTCA GGAAGTTGTC    120
GTGAAGCTGG CCGAGGAACT GATGCGCAAT GGTCAACTCG ACGACAGCTC GCCATTGGGA    180
AAACTGTTGG CCAAGTCGAT GGCCGCAGAT GGCAAGGCGG GCGGCGGTAT TGAGGATGTC    240
ATCGCTGCGC TGGACAAGCT GATCCATGAA AAGCTCGGTG ACAACTTCGG CGCGTCTGCG    300
GACAGCGCCT CGGGTACCGG ACAGCAGGAC CTGATGACTC AGGTGCTCAA TGGCCTGGCC    360
AAGTCGATGC TCGATGATCT TCTGACCAAG CAGGATGGCG GGACAAGCTT CTCCGAAGAC    420
GATATGCCGA TGCTGAACAA GATCGCGCAG TTCATGGATG ACAATCCCGC ACAGTTTCCC    480
AAGCCGGACT CGGGCTCCTG GGTGAACGAA CTCAAGGAAG ACAACTTCCT TGATGGCGAC    540
GAAACGGCTG CGTTCCGTTC GGCACTCGAC ATCATTGGCC AGCAACTGGG TAATCAGCAG    600
AGTGACGCTG GCAGTCTGGC AGGGACGGGT GGAGGTCTGG GCACTCCGAG CAGTTTTTCC    660
AACAACTCGT CCGTGATGGG TGATCCGCTG ATCGACGCCA ATACCGGTCC CGGTGACAGC    720
GGCAATACCC GTGGTGAAGC GGGGCAACTG ATCGGCGAGC TTATCGACCG TGGCCTGCAA    780
TCGGTATTGG CCGGTGGTGG ACTGGGCACA CCCGTAAACA CCCCGCAGAC CGGTACGTCG    840
GCGAATGGCG GACAGTCCGC TCAGGATCTT GATCAGTTGC TGGGCGGCTT GCTGCTCAAG    900
GGCCTGGAGG CAACGCTCAA GGATGCCGGG CAAACAGGCA CCGACGTGCA GTCGAGCGCT    960
GCGCAAATCG CCACCTTGCT GGTCAGTACG CTGCTGCAAG GCACCCGCAA TCAGGCTGCA   1020
GCCTGA                                                              1026
```

Another potentially suitable hypersensitive response elicitor from *Pseudomonas syringae* is disclosed in U.S. Patent Application Serial No. 09/120,817,

which is hereby incorporated by reference. The protein has a nucleotide sequence of SEQ. ID. No. 13 as follows:

TCCACTTCGC TGATTTTGAA ATTGGCAGAT TCATAGAAAC GTTCAGGTGT GGAAATCAGG 60

CTGAGTGCGC AGATTTCGTT GATAAGGGTG TGGTACTGGT CATTGTTGGT CATTTCAAGG 120

CCTCTGAGTG CGGTGCGGAG CAATACCAGT CTTCCTGCTG GCGTGTGCAC ACTGAGTCGC 180

AGGCATAGGC ATTTCAGTTC CTTGCGTTGG TTGGGCATAT AAAAAAAGGA ACTTTTAAAA 240

ACAGTGCAAT GAGATGCCGG CAAAACGGGA ACCGGTCGCT GCGCTTTGCC ACTCACTTCG 300

AGCAAGCTCA ACCCCAAACA TCCACATCCC TATCGAACGG ACAGCGATAC GGCCACTTGC 360

TCTGGTAAAC CCTGGAGCTG GCGTCGGTCC AATTGCCCAC TTAGCGAGGT AACGCAGCAT 420

GAGCATCGGC ATCACACCCC GGCCGCAACA GACCACCACG CCACTCGATT TTTCGGCGCT 480

AAGCGGCAAG AGTCCTCAAC CAAACACGTT CGGCGAGCAG AACACTCAGC AAGCGATCGA 540

CCCGAGTGCA CTGTTGTTCG GCAGCGACAC ACAGAAAGAC GTCAACTTCG GCACGCCCGA 600

CAGCACCGTC CAGAATCCGC AGGACGCCAG CAAGCCCAAC GACAGCCAGT CCAACATCGC 660

TAAATTGATC AGTGCATTGA TCATGTCGTT GCTGCAGATG CTCACCAACT CCAATAAAAA 720

GCAGGACACC AATCAGGAAC AGCCTGATAG CCAGGCTCCT TTCCAGAACA ACGGCGGGCT 780

CGGTACACCG TCGGCCGATA GCGGGGGCGG CGGTACACCG GATGCGACAG GTGGCGGCGG 840

CGGTGATACG CCAAGCGCAA CAGGCGGTGG CGGCGGTGAT ACTCCGACCG CAACAGGCGG 900

TGGCGGCAGC GGTGGCGGCG GCACACCCAC TGCAACAGGT GGCGGCAGCG GTGGCACACC 960

CACTGCAACA GGCGGTGGCG AGGGTGGCGT AACACCGCAA ATCACTCCGC AGTTGGCCAA 1020

CCCTAACCGT ACCTCAGGTA CTGGCTCGGT GTCGGACACC GCAGGTTCTA CCGAGCAAGC 1080

CGGCAAGATC AATGTGGTGA AAGACACCAT CAAGGTCGGC GCTGGCGAAG TCTTTGACGG 1140

CCACGGCGCA ACCTTCACTG CCGACAAATC TATGGGTAAC GGAGACCAGG GCGAAAATCA 1200

GAAGCCCATG TTCGAGCTGG CTGAAGGCGC TACGTTGAAG AATGTGAACC TGGGTGAGAA 1260

CGAGGTCGAT GGCATCCACG TGAAAGCCAA AAACGCTCAG GAAGTCACCA TTGACAACGT 1320

GCATGCCCAG AACGTCGGTG AAGACCTGAT TACGGTCAAA GGCGAGGGAG GCGCAGCGGT 1380

CACTAATCTG AACATCAAGA ACAGCAGTGC CAAAGGTGCA GACGACAAGG TTGTCCAGCT 1440

CAACGCCAAC ACTCACTTGA AAATCGACAA CTTCAAGGCC GACGATTTCG GCACGATGGT 1500

TCGCACCAAC GGTGGCAAGC AGTTTGATGA CATGAGCATC GAGCTGAACG GCATCGAAGC 1560

TAACCACGGC AAGTTCGCCC TGGTGAAAAG CGACAGTGAC GATCTGAAGC TGGCAACGGG 1620

CAACATCGCC ATGACCGACG TCAAACACGC CTACGATAAA ACCCAGGCAT CGACCCAACA 1680

CACCGAGCTT TGAATCCAGA CAAGTAGCTT GAAAAAAGGG GGTGGACTC 1729

This DNA molecule is known as the dspE gene for *Pseudomonas syringae.* This isolated DNA molecule of the present invention encodes a protein or polypeptide which elicits a plant pathogen's hypersensitive response having an amino acid sequence of SEQ. ID. No. 14 as follows:

```
Met Ser Ile Gly Ile Thr Pro Arg Pro Gln Gln Thr Thr Thr Pro Leu
1               5                   10                  15

Asp Phe Ser Ala Leu Ser Gly Lys Ser Pro Gln Pro Asn Thr Phe Gly
            20                  25                  30

Glu Gln Asn Thr Gln Gln Ala Ile Asp Pro Ser Ala Leu Leu Phe Gly
        35                  40                  45

Ser Asp Thr Gln Lys Asp Val Asn Phe Gly Thr Pro Asp Ser Thr Val
    50                  55                  60

Gln Asn Pro Gln Asp Ala Ser Lys Pro Asn Asp Ser Gln Ser Asn Ile
65                  70                  75                  80

Ala Lys Leu Ile Ser Ala Leu Ile Met Ser Leu Leu Gln Met Leu Thr
                85                  90                  95

Asn Ser Asn Lys Lys Gln Asp Thr Asn Gln Glu Gln Pro Asp Ser Gln
            100                 105                 110

Ala Pro Phe Gln Asn Asn Gly Gly Leu Gly Thr Pro Ser Ala Asp Ser
        115                 120                 125

Gly Gly Gly Gly Thr Pro Asp Ala Thr Gly Gly Gly Gly Gly Asp Thr
    130                 135                 140

Pro Ser Ala Thr Gly Gly Gly Gly Gly Asp Thr Pro Thr Ala Thr Gly
145                 150                 155                 160

Gly Gly Gly Ser Gly Gly Gly Gly Thr Pro Thr Ala Thr Gly Gly Gly
                165                 170                 175

Ser Gly Gly Thr Pro Thr Ala Thr Gly Gly Gly Glu Gly Gly Val Thr
            180                 185                 190

Pro Gln Ile Thr Pro Gln Leu Ala Asn Pro Asn Arg Thr Ser Gly Thr
        195                 200                 205

Gly Ser Val Ser Asp Thr Ala Gly Ser Thr Glu Gln Ala Gly Lys Ile
    210                 215                 220

Asn Val Val Lys Asp Thr Ile Lys Val Gly Ala Gly Glu Val Phe Asp
225                 230                 235                 240

Gly His Gly Ala Thr Phe Thr Ala Asp Lys Ser Met Gly Asn Gly Asp
                245                 250                 255

Gln Gly Glu Asn Gln Lys Pro Met Phe Glu Leu Ala Glu Gly Ala Thr
            260                 265                 270
```

```
Leu Lys Asn Val Asn Leu Gly Glu Asn Glu Val Asp Gly Ile His Val
        275                 280                 285

Lys Ala Lys Asn Ala Gln Glu Val Thr Ile Asp Asn Val His Ala Gln
    290                 295                 300

Asn Val Gly Glu Asp Leu Ile Thr Val Lys Gly Glu Gly Gly Ala Ala
305                 310                 315                     320

Val Thr Asn Leu Asn Ile Lys Asn Ser Ser Ala Lys Gly Ala Asp Asp
                325                 330                 335

Lys Val Val Gln Leu Asn Ala Asn Thr His Leu Lys Ile Asp Asn Phe
            340                 345                 350

Lys Ala Asp Asp Phe Gly Thr Met Val Arg Thr Asn Gly Gly Lys Gln
        355                 360                 365

Phe Asp Asp Met Ser Ile Glu Leu Asn Gly Ile Glu Ala Asn His Gly
    370                 375                 380

Lys Phe Ala Leu Val Lys Ser Asp Ser Asp Asp Leu Lys Leu Ala Thr
385                 390                 395                     400

Gly Asn Ile Ala Met Thr Asp Val Lys His Ala Tyr Asp Lys Thr Gln
                405                 410                 415

Ala Ser Thr Gln His Thr Glu Leu
            420
```

This protein or polypeptide is about 42.9 kDa.

This hypersensitive response elicitor from *Pseudomonas syringae* has 1 hypersensitive response eliciting domain. This domain extends, within SEQ. ID. No. 14, from amino acid 45 to amino acid 102, particularly from amino acid 58 to amino acid 92. The acidic unit in the first domain extends, within SEQ. ID. No. 14, from amino acid 45 to amino acid 79, particularly from amino acid 58 to amino acid 79. The alpha-helix in the first domain extends, within SEQ. ID. No. 14, from amino acid 79 to amino acid 102, particularly from amino acid 79 to amino acid 92.

The hypersensitive response elicitor polypeptide or protein derived from *Pseudomonas solanacearum* has an amino acid sequence corresponding to SEQ. ID. No. 15 as follows:

```
Met Ser Val Gly Asn Ile Gln Ser Pro Ser Asn Leu Pro Gly Leu Gln
1               5                   10                  15

Asn Leu Asn Leu Asn Thr Asn Thr Asn Ser Gln Gln Ser Gly Gln Ser
            20                  25                  30
```

```
Val Gln Asp Leu Ile Lys Gln Val Glu Lys Asp Ile Leu Asn Ile Ile
        35                  40                  45

Ala Ala Leu Val Gln Lys Ala Ala Gln Ser Ala Gly Gly Asn Thr Gly
    50                  55                  60

Asn Thr Gly Asn Ala Pro Ala Lys Asp Gly Asn Ala Asn Ala Gly Ala
65                  70                  75                  80

Asn Asp Pro Ser Lys Asn Asp Pro Ser Lys Ser Gln Ala Pro Gln Ser
                85                  90                  95

Ala Asn Lys Thr Gly Asn Val Asp Asp Ala Asn Asn Gln Asp Pro Met
            100                 105                 110

Gln Ala Leu Met Gln Leu Leu Glu Asp Leu Val Lys Leu Leu Lys Ala
        115                 120                 125

Ala Leu His Met Gln Gln Pro Gly Gly Asn Asp Lys Gly Asn Gly Val
    130                 135                 140

Gly Gly Ala Asn Gly Ala Lys Gly Ala Gly Gly Gln Gly Gly Leu Ala
145                 150                 155                 160

Glu Ala Leu Gln Glu Ile Glu Gln Ile Leu Ala Gln Leu Gly Gly Gly
                165                 170                 175

Gly Ala Gly Ala Gly Gly Ala Gly Gly Gly Val Gly Gly Ala Gly Gly
            180                 185                 190

Ala Asp Gly Gly Ser Gly Ala Gly Gly Ala Gly Gly Ala Asn Gly Ala
        195                 200                 205

Asp Gly Gly Asn Gly Val Asn Gly Asn Gln Ala Asn Gly Pro Gln Asn
    210                 215                 220

Ala Gly Asp Val Asn Gly Ala Asn Gly Ala Asp Asp Gly Ser Glu Asp
225                 230                 235                 240

Gln Gly Gly Leu Thr Gly Val Leu Gln Lys Leu Met Lys Ile Leu Asn
                245                 250                 255

Ala Leu Val Gln Met Met Gln Gln Gly Gly Leu Gly Gly Gly Asn Gln
            260                 265                 270

Ala Gln Gly Gly Ser Lys Gly Ala Gly Asn Ala Ser Pro Ala Ser Gly
        275                 280                 285

Ala Asn Pro Gly Ala Asn Gln Pro Gly Ser Ala Asp Asp Gln Ser Ser
    290                 295                 300

Gly Gln Asn Asn Leu Gln Ser Gln Ile Met Asp Val Val Lys Glu Val
305                 310                 315                 320

Val Gln Ile Leu Gln Gln Met Leu Ala Ala Gln Asn Gly Gly Ser Gln
                325                 330                 335
```

```
Gln Ser Thr Ser Thr Gln Pro Met
            340
```

It is encoded by a DNA molecule having a nucleotide sequence corresponding SEQ. ID. No. 16 as follows:

```
ATGTCAGTCG GAAACATCCA GAGCCCGTCG AACCTCCCGG GTCTGCAGAA CCTGAACCTC     60
AACACCAACA CCAACAGCCA GCAATCGGGC CAGTCCGTGC AAGACCTGAT CAAGCAGGTC    120
GAGAAGGACA TCCTCAACAT CATCGCAGCC CTCGTGCAGA AGGCCGCACA GTCGGCGGGC    180
GGCAACACCG GTAACACCGG CAACGCGCCG GCGAAGGACG GCAATGCCAA CGCGGGCGCC    240
AACGACCCGA GCAAGAACGA CCCGAGCAAG AGCCAGGCTC CGCAGTCGGC CAACAAGACC    300
GGCAACGTCG ACGACGCCAA CAACCAGGAT CCGATGCAAG CGCTGATGCA GCTGCTGGAA    360
GACCTGGTGA AGCTGCTGAA GGCGGCCCTG CACATGCAGC AGCCCGGCGG CAATGACAAG    420
GGCAACGGCG TGGGCGGTGC CAACGGCGCC AAGGGTGCCG GCGGCCAGGG CGGCCTGGCC    480
GAAGCGCTGC AGGAGATCGA GCAGATCCTC GCCCAGCTCG GCGGCGGCGG TGCTGGCGCC    540
GGCGGCGCGG GTGGCGGTGT CGGCGGTGCT GGTGGCGCGG ATGGCGGCTC CGGTGCGGGT    600
GGCGCAGGCG GTGCGAACGG CGCCGACGGC GGCAATGGCG TGAACGGCAA CCAGGCGAAC    660
GGCCCGCAGA ACGCAGGCGA TGTCAACGGT GCCAACGGCG CGGATGACGG CAGCGAAGAC    720
CAGGGCGGCC TCACCGGCGT GCTGCAAAAG CTGATGAAGA TCCTGAACGC GCTGGTGCAG    780
ATGATGCAGC AAGGCGGCCT CGGCGGCGGC AACCAGGCGC AGGGCGGCTC GAAGGGTGCC    840
GGCAACGCCT CGCCGGCTTC CGGCGCGAAC CCGGGCGCGA ACCAGCCCGG TTCGGCGGAT    900
GATCAATCGT CCGGCCAGAA CAATCTGCAA TCCCAGATCA TGGATGTGGT GAAGGAGGTC    960
GTCCAGATCC TGCAGCAGAT GCTGGCGGCG CAGAACGGCG GCAGCCAGCA GTCCACCTCG   1020
ACGCAGCCGA TGTAA                                                   1035
```

Further information regarding the hypersensitive response elicitor polypeptide or protein derived from *Pseudomonas solanacearum* is set forth in Arlat, M., F. Van Gijsegem, J. C. Huet, J. C. Pemollet, and C. A. Boucher, "PopA1, a Protein which Induces a Hypersensitive-like Response in Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-533 (1994), which is hereby incorporated by reference.

The hypersensitive response elicitor from *Pseudomonas solanacearum* has 2 hypersensitive response eliciting domains. The first domain extends, within SEQ. ID.

No. 15, from amino acid 85 to amino acid 131, particularly from amino acid 95 to amino acid 123. The acidic unit in the first domain extends, within SEQ. ID. No. 15, from amino acid 85 to amino acid 111, particularly from amino acid 95 to amino acid 123. The alpha-helix in the first domain extends, within SEQ. ID. No. 15, from amino acid 85 to amino acid 111, particularly from amino acid 95 to amino acid 111. The second domain extends, within SEQ. ID. No. 15, from amino acid 195 to amino acid 264, particularly from amino acid 229 to amino acid 258. The acidic unit in the second domain extends, within SEQ. ID. No. 15, from amino acid 195 to amino acid 246, particularly from amino acid 229 to amino acid 264. The alpha-helix in the second domain extends, within SEQ. ID. No. 15, from amino acid 246 to amino acid 264, particularly from amino acid 246 to amino acid 258.

The N-terminus of the hypersensitive response elicitor polypeptide or protein from *Xanthomonas campestris* has an amino acid sequence corresponding to SEQ. ID. No. 17 as follows:

```
Met Asp Gly Ile Gly Asn His Phe Ser Asn
  1               5                  10
```

The hypersensitive response elicitor polypeptide or protein from *Xanthomonas campestris pv. pelargonii* is heat stable, protease sensitive, and has a molecular weight of 20 kDa. It includes an amino acid sequence corresponding to SEQ. ID. No. 18 as follows:

```
Ser Ser Gln Gln Ser Pro Ser Ala Gly Ser Glu Gln Gln Leu Asp Gln
  1               5                  10                  15

Leu Leu Ala Met
             20
```

Isolation of *Erwinia carotovora* hypersensitive response elictor protein or polypeptide is described in Cui et al., "The RsmA Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrp* N_{Ecc} and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI, 9(7):565-73 (1996), which is hereby incorporated by reference. The hypersensitive response elicitor protein or polypeptide of *Erwinia stewartii* is set forth in Ahmad et al., "Harpin is Not

Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," 8th Int'l. Cong. Molec. Plant-Microbe Interact., July 14-19, 1996 and Ahmad, et al., "Harpin is Not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," Ann. Mtg. Am. Phytopath. Soc., July 27-31, 1996, which are hereby incorporated by reference.

Hypersensitive response elicitor proteins or polypeptides from *Phytophthora parasitica, Phytophthora cryptogea, Phytophthora cinnamoni, Phytophthora capsici, Phytophthora megasperma,* and *Phytophora citrophthora* are described in Kaman, et al., "Extracellular Protein Elicitors from Phytophthora: Most Specificity and Induction of Resistance to Bacterial and Fungal Phytopathogens," Molec. Plant-Microbe Interact., 6(1):15-25 (1993), Ricci et al., "Structure and Activity of Proteins from Pathogenic Fungi Phytophthora Eliciting Necrosis and Acquired Resistance in Tobacco," Eur. J. Biochem., 183:555-63 (1989), Ricci et al., "Differential Production of Parasiticein, and Elicitor of Necrosis and Resistance in Tobacco, by Isolates of Phytophthora parasitica," Plant Path. 41:298-307 (1992), Baillreul et al, "A New Elicitor of the Hypersensitive Response in Tobacco: A Fungal Glycoprotein Elicits Cell Death, Expression of Defence Genes, Production of Salicylic Acid, and Induction of Systemic Acquired Resistance," Plant J., 8(4):551-60 (1995), and Bonnet et al., "Acquired Resistance Triggered by Elicitors in Tobacco and Other Plants," Eur. J. Plant Path., 102:181-92 (1996), which are hereby incorporated by reference.

Another hypersensitive response elicitor in accordance with the present invention is from *Clavibacter michiganensis* subsp. *sepedonicus* which is fully described in U.S. Patent Application Serial No. 09/136,625, which is hereby incorporated by reference.

The above elicitors are exemplary. Other elicitors can be identified by growing fungi or bacteria that elicit a hypersensitive response under conditions which genes encoding an elicitor are expressed. Cell-free preparations from culture supernatants can be tested for elicitor activity (i.e. local necrosis) by using them to infiltrate appropriate plant tissues.

Fragments of the above hypersensitive response elicitor polypeptides or proteins as well as fragments of full length elicitors from other pathogens are encompassed by the method of the present invention.

Suitable fragments can be produced by several means. In the first, subclones of the gene encoding a known elicitor protein are produced by conventional molecular genetic manipulation by subcloning gene fragments. The subclones then are expressed *in vitro* or *in vivo* in bacterial cells to yield a smaller protein or peptide that can be tested for elicitor activity according to the procedure described below.

As an alternative, fragments of an elicitor protein can be produced by digestion of a full-length elicitor protein with proteolytic enzymes like chymotrypsin or *Staphylococcus* proteinase A, or trypsin. Different proteolytic enzymes are likely to cleave elicitor proteins at different sites based on the amino acid sequence of the elicitor protein. Some of the fragments that result from proteolysis may be active elicitors of resistance.

In another approach, based on knowledge of the primary structure of the protein, fragments of the elicitor protein gene may be synthesized by using the PCR technique together with specific sets of primers chosen to represent particular portions of the protein. These then would be cloned into an appropriate vector for expression of a truncated peptide or protein.

Chemical synthesis can also be used to make suitable fragments. Such a synthesis is carried out using known amino acid sequences for the elicitor being produced. Alternatively, subjecting a full length elicitor to high temperatures and pressures will produce fragments. These fragments can then be separated by conventional procedures (e.g., chromatography, SDS-PAGE).

An example of suitable fragments of a hypersensitive response elicitor which do elicit a hypersensitive response are *Erwinia amylovora* fragments including a C-terminal fragment of the amino acid sequence of SEQ. ID. No. 3, an N-terminal fragment of the amino acid sequence of SEQ. ID. No. 3, or an internal fragment of the amino acid sequence of SEQ. ID. No. 3. The C-terminal fragment of the amino acid sequence of SEQ. ID. No. 3 can span amino acids 105 and 403 of SEQ. ID. No. 3. The N-terminal fragment of the amino acid sequence of SEQ. ID. No. 3 can span the following amino acids of SEQ. ID. No. 3: 1 and 98, 1 and 104, 1 and 122, 1 and 168, 1 and 218, 1 and 266, 1 and 342, 1 and 321, and 1 and 372. The internal fragment of the amino acid sequence of SEQ. ID. No. 3 can span the following amino acids of

SEQ. ID. No. 3: 76 and 209, 105 and 209, 99 and 209, 137 and 204, 137 and 200, 109 and 204, 109 and 200, 137 and 180, and 105 and 180.

Suitable DNA molecules are those that hybridize to the DNA molecule comprising a nucleotide sequence of SEQ. ID. Nos. 2, 4, 5, 7, 9, 12, 13, and 16 under stringent conditions. An example of suitable high stringency conditions is when hybridization is carried out at 65°C for 20 hours in a medium containing 1M NaCl, 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.1% sodium dodecyl sulfate, 0.2% ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 50 μm g/ml *E. coli* DNA. Suitable stringency conditions also include hybridization in a hybridization buffer comprising 0.9M sodium citrate ("SSC") buffer at a temperature of 37°C where hybridized nucleic acids remain bound when subject to washing the SSC buffer at a temperature of 37°C; and preferably in a hybridization buffer comprising 20% formamide in 0.9M SSC buffer at a temperature of 42°C where hybridized nucleic acids remain bound when subject to washing at 42°C with 0.2x SSC buffer at 42°C.

Variants may be made by, for example, the deletion or addition of amino acids that have minimal influence on the properties, secondary structure and hydropathic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the polypeptide.

A particularly advantageous aspect of the present invention involves utilizing a protein having a pair or more, particularly 3 or more, coupled domains. These domains can be from different source organisms. When a DNA molecule encoding such a protein is prepared, it can be advantageously used to make transgenic plants. The use of a gene encoding such domains, as opposed to a gene encoding a full length hypersensitive response elicitor, has a number of benefits. Firstly, such a gene is easier to synthesize. More significantly, the use of a plurality of domains together from different source organisms can impart their combined benefits to a transgenic plant.

The DNA molecule encoding the hypersensitive response elicitor polypeptide or protein can be incorporated in cells using conventional recombinant

DNA technology. Generally, this involves inserting the DNA molecule into an expression system to which the DNA molecule is heterologous (i.e. not normally present). The heterologous DNA molecule is inserted into the expression system or vector in proper sense orientation and correct reading frame. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences.

U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.

Recombinant genes may also be introduced into viruses, such as vaccina virus. Recombinant viruses can be generated by transfection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC1084, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see F.W. Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," <u>Gene Expression Technology</u> vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook et al., <u>Molecular Cloning: A Laboratory Manual</u>, Cold Springs Laboratory, Cold Springs Harbor, New York (1989), which is hereby incorporated by reference.

A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria

transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

Transcription of DNA is dependent upon the presence of a promotor which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promotors differ from those of procaryotic promotors. Furthermore, eucaryotic promotors and accompanying genetic signals may not be recognized in or may not function in a procaryotic system, and, further, procaryotic promotors are not recognized and do not function in eucaryotic cells.

Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes. Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473 (1979), which is hereby incorporated by reference.

Promotors vary in their "strength" (i.e. their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promotors in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promotors may be used. For instance, when cloning in *E. coli*, its

bacteriophages, or plasmids, promotors such as the T7 phage promoter, *lac* promotor, *trp* promotor, *rec*A promotor, ribosomal RNA promotor, the P_R and P_L promotors of coliphage lambda and others, including but not limited, to *lac*UV5, *omp*F, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lac*UV5 (*tac*) promotor or other *E. coli* promotors produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promotor unless specifically induced. In certain operations, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the *lac* operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different controls.

Specific initiation signals are also required for efficient gene transcription and translation in procaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promotor, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires an SD sequence about 7-9 bases 5' to the initiation codon ("ATG") to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *cro* gene or the *N* gene of coliphage lambda, or from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Once the isolated DNA molecule encoding the hypersensitive response elicitor polypeptide or protein has been cloned into an expression system, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but are not limited to, plant cells as well as

prokaryotic and eukaryotic cells, such as bacteria, virus, yeast, mammalian, insect cells, and the like.

The present invention further relates to methods of imparting disease resistance to plants, enhancing plant growth, effecting insect control and/or imparting stress resistance to plants. These methods involve applying a hypersensitive response elicitor polypeptide or protein to all or part of a plant or a plant seed under conditions where the polypeptide or protein contacts all or part of the cells of the plant or plant seed. Alternatively, the hypersensitive response elicitor protein or polypeptide can be applied to plants such that seeds recovered from such plants themselves are able to impart disease resistance in plants, to enhance plant growth, to effect insect control, and/or to impart stress resistance.

As an alternative to applying a hypersensitive response elicitor polypeptide or protein to plants or plant seeds in order to impart disease resistance in plants, to effect plant growth, to control insects, and/or to impart stress resistance to the plants or plants grown from the seeds, transgenic plants or plant seeds can be utilized. When utilizing transgenic plants, this involves providing a transgenic plant transformed with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein and growing the plant under conditions effective to permit that DNA molecule to impart disease resistance to plants, to enhance plant growth, to control insects, and/or to impart stress resistance. Alternatively, a transgenic plant seed transformed with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein can be provided and planted in soil. A plant is then propagated from the planted seed under conditions effective to permit that DNA molecule to impart disease resistance to plants, to enhance plant growth, to control insects, and/or to impart stress resistance.

The method of the present invention can be utilized to treat a wide variety of plants or their seeds to impart disease resistance, enhance growth, control insects, and/or to impart stress resistance. Suitable plants include dicots and monocots. More particularly, useful crop plants can include: alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, turnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash,

pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane. Examples of suitable ornamental plants are: *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

With regard to the use of the hypersensitive response elicitor protein or polypeptide of the present invention in imparting disease resistance, absolute immunity against infection may not be conferred, but the severity of the disease is reduced and symptom development is delayed. Lesion number, lesion size, and extent of sporulation of fungal pathogens are all decreased. This method of imparting disease resistance has the potential for treating previously untreatable diseases, treating diseases systemically which might not be treated separately due to cost, and avoiding the use of infectious agents or environmentally harmful materials.

The method of imparting pathogen resistance to plants in accordance with the present invention is useful in imparting resistance to a wide variety of pathogens including viruses, bacteria, and fungi. Resistance, *inter alia*, to the following viruses can be achieved by the method of the present invention: *Tobacco mosaic virus* and *Tomato mosaic virus*. Resistance, *inter alia*, to the following bacteria can also be imparted to plants in accordance with present invention: *Pseudomonas solancearum*, *Pseudomonas syringae* pv. *tabaci*, and *Xanthamonas campestris* pv. *pelargonii*. Plants can be made resistant, *inter alia*, to the following fungi by use of the method of the present invention: *Fusarium oxysporum* and *Phytophthora infestans*.

With regard to the use of the hypersensitive response elicitor protein or polypeptide of the present invention to enhance plant growth, various forms of plant growth enhancement or promotion can be achieved. This can occur as early as when plant growth begins from seeds or later in the life of a plant. For example, plant growth according to the present invention encompasses greater yield, increased quantity of seeds produced, increased percentage of seeds germinated, increased plant size, greater biomass, more and bigger fruit, earlier fruit coloration, and earlier fruit and plant maturation. As a result, the present invention provides significant economic benefit to growers. For example, early germination and early maturation permit crops to be grown in areas where short growing seasons would otherwise preclude their

growth in that locale. Increased percentage of seed germination results in improved crop stands and more efficient seed use. Greater yield, increased size, and enhanced biomass production allow greater revenue generation from a given plot of land.

Another aspect of the present invention is directed to effecting any form of insect control for plants. For example, insect control according to the present invention encompasses preventing insects from contacting plants to which the hypersensitive response elicitor has been applied, preventing direct insect damage to plants by feeding injury, causing insects to depart from such plants, killing insects proximate to such plants, interfering with insect larval feeding on such plants, preventing insects from colonizing host plants, preventing colonizing insects from releasing phytotoxins, etc. The present invention also prevents subsequent disease damage to plants resulting from insect infection.

The present invention is effective against a wide variety of insects. European corn borer is a major pest of corn (dent and sweet corn) but also feeds on over 200 plant species including green, wax, and lima beans and edible soybeans, peppers, potato, and tomato plus many weed species. Additional insect larval feeding pests which damage a wide variety of vegetable crops include the following: beet armyworm, cabbage looper, corn ear worm, fall armyworm, diamondback moth, cabbage root maggot, onion maggot, seed corn maggot, pickleworm (melonworm), pepper maggot, and tomato pinworm. Collectively, this group of insect pests represents the most economically important group of pests for vegetable production worldwide.

Another aspect of the present invention is directed to imparting stress resistance to plants. Stress encompasses any environmental factor having an adverse effect on plant physiology and development. Examples of such environmental stress include climate-related stress (e.g., drought, water, frost, cold temperature, high temperature, excessive light, and insufficient light), air polllution stress (e.g., carbon dioxide, carbon monoxide, sulfur dioxide, NO_x, hydrocarbons, ozone, ultraviolet radiation, acidic rain), chemical (e.g., insecticides, fungicides, herbicides, heavy metals), and nutritional stress (e.g., fertilizer, micronutrients, macronutrients). Use of hypersensitive response elicitors in accordance with the present invention impart resistance to plants against such forms of environmental stress.

The method of the present invention involving application of the hypersensitive response elicitor polypeptide or protein can be carried out through a variety of procedures when all or part of the plant is treated, including leaves, stems, roots, propagules (e.g., cuttings), etc. This may (but need not) involve infiltration of the hypersensitive response elicitor polypeptide or protein into the plant. Suitable application methods include high or low pressure spraying, injection, and leaf abrasion proximate to when elicitor application takes place. When treating plant seeds, in accordance with the application embodiment of the present invention, the hypersensitive response elicitor protein or polypeptide can be applied by low or high pressure spraying, coating, immersion, or injection. Other suitable application procedures can be envisioned by those skilled in the art provided they are able to effect contact of the hypersensitive response elicitor polypeptide or protein with cells of the plant or plant seed. Once treated with the hypersensitive response elicitor of the present invention, the seeds can be planted in natural or artificial soil and cultivated using conventional procedures to produce plants. After plants have been propagated from seeds treated in accordance with the present invention, the plants may be treated with one or more applications of the hypersensitive response elicitor protein or polypeptide to impart disease resistance to plants, to enhance plant growth, to control insects on the plants, and/or impart stress resistance.

The hypersensitive response elicitor polypeptide or protein can be applied to plants or plant seeds in accordance with the present invention alone or in a mixture with other materials. Alternatively, the hypersensitive response elicitor polypeptide or protein can be applied separately to plants with other materials being applied at different times.

A composition suitable for treating plants or plant seeds in accordance with the application embodiment of the present invention contains a hypersensitive response elicitor polypeptide or protein in a carrier. Suitable carriers include water, aqueous solutions, slurries, or dry powders. In this embodiment, the composition contains greater than 500 nM hypersensitive response elicitor polypeptide or protein.

Although not required, this composition may contain additional additives including fertilizer, insecticide, fungicide, nematacide, and mixtures thereof.

Suitable fertilizers include $(NH_4)_2NO_3$. An example of a suitable insecticide is Malathion. Useful fungicides include Captan.

Other suitable additives include buffering agents, wetting agents, coating agents, and abrading agents. These materials can be used to facilitate the process of the present invention. In addition, the hypersensitive response elicitor polypeptide or protein can be applied to plant seeds with other conventional seed formulation and treatment materials, including clays and polysaccharides.

In the alternative embodiment of the present invention involving the use of transgenic plants and transgenic seeds, a hypersensitive response elicitor polypeptide or protein need not be applied topically to the plants or seeds. Instead, transgenic plants transformed with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein are produced according to procedures well known in the art.

The vector described above can be microinjected directly into plant cells by use of micropipettes to transfer mechanically the recombinant DNA. Crossway, Mol. Gen. Genetics, 202:179-85 (1985), which is hereby incorporated by reference. The genetic material may also be transferred into the plant cell using polyethylene glycol. Krens, et al., Nature, 296:72-74 (1982), which is hereby incorporated by reference.

Another approach to transforming plant cells with a gene which imparts resistance to pathogens is particle bombardment (also known as biolistic transformation) of the host cell. This can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., which are hereby incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the heterologous DNA. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g.,

dried bacterial cells containing the vector and heterologous DNA) can also be propelled into plant cells.

Yet another method of introduction is fusion of protoplasts with other entities, either minicells, cells, lysosomes or other fusible lipid-surfaced bodies. Fraley, et al., Proc. Natl. Acad. Sci. USA, 79:1859-63 (1982), which is hereby incorporated by reference.

The DNA molecule may also be introduced into the plant cells by electroporation. Fromm et al., Proc. Natl. Acad. Sci. USA, 82:5824 (1985), which is hereby incorporated by reference. In this technique, plant protoplasts are electroporated in the presence of plasmids containing the expression cassette. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and regenerate.

Another method of introducing the DNA molecule into plant cells is to infect a plant cell with *Agrobacterium tumefaciens* or *A. rhizogenes* previously transformed with the gene. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots or roots, and develop further into plants. Generally, this procedure involves inoculating the plant tissue with a suspension of bacteria and incubating the tissue for 48 to 72 hours on regeneration medium without antibiotics at 25-28°C.

Agrobacterium is a representative genus of the gram-negative family Rhizobiaceae. Its species are responsible for crown gall (*A. tumefaciens*) and hairy root disease (*A. rhizogenes*). The plant cells in crown gall tumors and hairy roots are induced to produce amino acid derivatives known as opines, which are catabolized only by the bacteria. The bacterial genes responsible for expression of opines are a convenient source of control elements for chimeric expression cassettes. In addition, assaying for the presence of opines can be used to identify transformed tissue.

Heterologous genetic sequences can be introduced into appropriate plant cells, by means of the Ti plasmid of *A. tumefaciens* or the Ri plasmid of *A. rhizogenes*. The Ti or Ri plasmid is transmitted to plant cells on infection by Agrobacterium and is stably integrated into the plant genome. J. Schell, Science, 237:1176-83 (1987), which is hereby incorporated by reference.

After transformation, the transformed plant cells must be regenerated.

Plant regeneration from cultured protoplasts is described in Evans et al., Handbook of Plant Cell Cultures, Vol. 1: (MacMillan Publishing Co., New York, 1983); and Vasil I.R. (ed.), Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, Orlando, Vol. I, 1984, and Vol. III (1986), which are hereby incorporated by reference.

It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to, all major species of sugarcane, sugar beets, cotton, fruit trees, and legumes.

Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced in the callus tissue. These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is usually reproducible and repeatable.

After the expression cassette is stably incorporated in transgenic plants, it can be transferred to other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

Once transgenic plants of this type are produced, the plants themselves can be cultivated in accordance with conventional procedure with the presence of the gene encoding the hypersensitive response elicitor resulting in disease resistance, enhanced plant growth, control of insects on the plant, and/or stress resistance. Alternatively, transgenic seeds are recovered from the transgenic plants. These seeds can then be planted in the soil and cultivated using conventional procedures to produce transgenic plants. The transgenic plants are propagated from the planted transgenic seeds under conditions effective to impart disease resistance to plants, to enhance plant growth, to control insects, and/or to impart stress resistance. While not

wishing to be bound by theory, such disease resistance, growth enhancement, insect control, and/or stress resistance may be RNA mediated or may result from expression of the elicitor polypeptide or protein.

When transgenic plants and plant seeds are used in accordance with the present invention, they additionally can be treated with the same materials as are used to treat the plants and seeds to which a hypersensitive response elicitor polypeptide or protein is applied. These other materials, including hypersensitive response elicitors, can be applied to the transgenic plants and plant seeds by the above-noted procedures, including high or low pressure spraying, injection, coating, and immersion. Similarly, after plants have been propagated from the transgenic plant seeds, the plants may be treated with one or more applications of the hypersensitive response elicitor to impart disease resistance, enhance growth, control insects, and/or to impart stress resistance. Such plants may also be treated with conventional plant treatment agents (e.g., insecticides, fertilizers, etc.).

EXAMPLES

<u>Example 1</u> - Bacterial Strains and Plasmids

Escherichia coli DH5 and BL21 were purchased from Gibco BRL (Rockville, MD) and Novagen (Madison, WI) respectively.

pET28 plasmids were from Novagen (Madison, WI).

All restriction enzymes (e.g., NdeI and HindIII), T4 DNA ligase, Calf intestinal alkaline phosphatase (CIP), and PCR reagents were from Gibco BRL (Rockville, MD).

Oligonucleotides were synthesized by Lofstrand Labs Ltd (Gaithersburg, MD).

Chemically synthesized polypeptides were synthesized by Bio-Synthesis (Lewisville, TX).

<u>Example 2</u> - Construction of Truncated Gene Encoding Harpin

Fragments of genes encoding harpin proteins were constructed in pET28 vector and expressed in *E. coli* as follows;

1. HrpN fragments were PCR amplified from the pCPP2139 plasmid (Cornell University, Ithaca, NY) and cloned into pET28 vector.
2. HrpZ fragments were PCR amplified from the pSYH10 plasmid (Cornell University, Ithaca, NY) and cloned into pET28 vector.
3. PopA fragments were PCR amplified from the pBS::popA plasmid (Cornell University, Ithaca, NY) and cloned into pET28 vector.
4. HrpW fragments were PCR amplified from the pCPP1233 plasmid (Cornell University, Ithaca, NY) and cloned into pET28 vector.

All truncated fragments were amplified by PCR with full length harpin DNA as the template.

Oligonucleotides corresponding to the truncated N-terminal sequence were started /modified with a Nde I site (which serves as an initiation codon of methionine (ATG)). Oligonucleotides corresponding to a C-terminal sequence contained a UAA stop codon followed by a Hind III site.

PCR was carried in a 0.5 ml tube with GeneAmpTM 9600 and 9700 (PE Applied Biosystems, Branchburg, New Jersey). 45 µl of SuperMix™ (Gibco BRL, Rockville, MD) was mixed with 20 pmoles of each pair of DNA primers, 10 ng of full length harpin DNA, and diH_2O to fill the final volume to 50 µl. After heating the mixture at 95°C for 2 min., PCR was performed for 30 cycles at 94°C for 1 min., 58°C for 1 min. and 72°C for 1.5 min. Amplified DNAs were purified with QIAquick PCR purification kit (QIAGEN Inc., Vlencia, CA), digested with Nde I and Hind III at 37°C for 5 hours, extracted once with phenol:chloroform:isoamylalcohol (25:24:1), and precipitated with ethanol. 5 µg of pET28(b) vector DNA was digested with 15 units of Nde I and 20 units of Hind III at 37°C for 3 hours followed with calf intestinal alkaline phosphatase treatment for 30 min. at 37°C to reduce the background resulting from incomplete single enzyme digestion. Digested vector DNA was purified with the QIAquick PCR purification kit and directly used for ligation. Ligation was carried at 14°C for 12 hours in a 15 µl mixture containing about 50 to

100 ng of digested pET28(b), 10 to 30 ng of targeted PCR fragments, and 1 unit of T4 DNA ligase. 5 μl of ligation solution was added to 100 μl of DH5α/XL1-Blue competent cells, placed in 15 ml Falcon tube, and incubated on ice for 30 min. After heat shock at 42°C for 45 seconds, 0.9 ml SOC solution (20 g bacto-tryptone, 5 g bacto-yeast extracts, 0.5 g NaCl, 20 mM glucose in one liter) was added into the tube and incubated at 37°C for 1 hour. 20 μl of transformed cells were plated onto LB agar plate with 30 μg/ml of kanamycin and incubated at 37°C for 14 hours. Single colonies were transferred to 3 ml LB-media and incubated overnight at 37°C. Plasmid DNA was prepared in a 2 ml culture with QIAprep Miniprep kit according to the manufacture's instruction. The DNA sequence of truncated harpin constructions was verified with restriction enzyme analysis and sequencing analysis. Plasmids with the desired DNA sequence were transferred into the BL21 strain with a standard chemical transformation method as indicated above.

<u>Example 3</u> - Expression of Proteins

A single clone of *E. coli* with a constructed gene was grown overnight at 37°C in LB with kanamycin. A proper amount of overnight culture was transferred to 50 to 500 ml LB and incubated at 37°C until OD600 reached 0.5 to 0.8. ITPG was added to the culture which was further incubated at room temperature for a period of 5 hour to overnight. Alternatively, a proper amount of overnight culture was transferred to 50 to 500 ml of ½ TB with lactose medium (6 g bacto-trypton, 12 g bacto-yeast extract, 75 g lactose in one liter). After incubation at 37°C until the OD600 reached 0.5 to 0.8, the culture was incubated at room temperature for a period of 5 hours to overnight.

All bacterial cells were harvested by centrifugation and resuspended in 1:5 TE buffer (10 mM Tris, pH 8.5 and 1 mM EDTA). The cells were disrupted by sonication and clarified by centrifugation. Supernatants were then infiltrated into tobacco leaves for HR testing.

Heat treatment (i.e. boiling for 1 to 10 min.) was used to achieve further purification.

All truncated fragments of genes encoding harpin protein were expressed in *E. coli*/ BL-21, DE3 strain with an N-terminal His-tag and 20 to 21

amino acid residues generated from the expression vector sequence. The His-tag sequence did not affect the HR activity of the proteins. In some cases, Ni-Agarose beads were added into supernatant solution and mixed at 4°C to room temperature for a period of 30 min. to overnight. The proteins bound to the Ni-Agarose beads were washed by 0.1 M imidazole buffer, and proteins were eluted with 0.6 to 1.0 M imidazole. After dialysis against 10 mM Tris, pH 8.5 buffer, the proteins were infiltrated into tobacco leaves for HR testing.

For proteins expressed in *E. coli* that were difficult to dissolve in water, total cells were resuspended and sonicated in 8 M urea buffer (0.1M Na-phosphate, 10 mM Tris buffer, pH8.0). The total cell lysate was centrifuged, and supernatants were collected. Ni-agarose was added into the supernatants and mixed gently at room temperature for 30 min. The Ni-agarose resin was washed with buffer (8 M urea, 0.1 M Na-phosphate, 10 mM Tris buffer, pH6.3). The proteins were eluted with elution buffer (8 M urea, 0.1 M EDTA, 0.1 M Na-phosphate, 10 mM Tris buffer, pH 6.3) and dialyzed against buffer (pH 8.5, 10 mM Tris) with stepwise decreased urea. If the proteins still were insoluble in buffer, the solution pH was adjusted to 9 to 11 and sonicated at room temperature for 1 to 5 min.

Chemically synthesized polypeptides were dissolved in 10 mM Tris, pH 6.5 to 11 buffers depending on their solubility.

A hypersensitive response ("HR") assay was performed by infiltration of 0.1 to 0.3 ml of serial diluted protein solutions into tobacco leaves (cv. Xanth). All HR data shown in these examples were recorded from 48 hours after infiltration.

Example 4 - Quantification of Proteins

All expressed proteins were checked with pre-cast 4-20% SDS polyacrylamide gel electrophoresis (SDS-PAGE) from Novex (San Diego, CA). After electrophoresis, the gel was stained with Coomasssie R-250 solution (0.1% Coomassie R-250, 10% Acetate Acid, 40% ethanol) for 1 to 4 hours and distained with distaining solution (8% acetate acid and 25% ethanol) overnight. The density of corresponding bands were compared to standard proteins, which were either purchased from Novex or were from quantitative standard harpin protein produced by Eden Bioscience (Bothell, Washington).

Example 5 - Classification of Harpin Proteins

Since harpin proteins share common biochemical and biophysical characteristics as well as biological functions, based on their unique properties, HR elicitors from various pathogenic bacteria should be viewed as belonging to a new protein family—i.e. the harpin protein family. The harpin protein can be classified into at least four subfamilies based on their primary structure and isolated sources. As set forth in Table 1, those subfamilies are identified by the designation N, W, Z, A, etc.

Table 1 - Subfamilies of Harpin Proteins

Harpin proteins	Isolated Source	Classified Subfamily	pI	Amino acids	Heat stable	Core structure
$HrpN_{Ea}$	*E. amylovora*	N	4.42	403	Yes	No
$HrpN_{Ech}$	*E. chrysanthemi*	N	6.51	340	Yes	No
$HrpN_{Ecc}$	*E. carotovora*	N	5.82	356	Yes	No
$HrpN_{Est}$	*E. stewartii*	N	N/A	N/A	Yes	No
$HrpW_{Pss}$	*P. syringae*	W	4.43	424	Yes	No
$HrpW_{Ea}$	*E. amylovora*	W	4.46	447	Yes	No
$HrpZ_{Pss}$	*P. syringae*	Z	3.95	341	Yes	No
PopA1	*R.solanacearum*	A	4.16	344	Yes	No

Example 6 - Analysis of the Structural Units of an HR Domain

The sequence of amino acids that alone could elicit a hypersensitive response in plants (i.e. HR domains) has been investigated in different ways. It was reported that a carboxyl-terminal 148 amino acid portion of $HrpZ_{Pss}$ is sufficient and necessary for HR (He et al., "Pseudomonas Syringae pv. Syringae $Harpin_{pss}$: A Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," Cell 73:1255-1266.(1993), which is hereby incorporated by reference). With truncated HrpZ fragments, it was determined that an N-terminal 109 amino acids and C-terminal 216 amino acids of $HrpZ_{Pss}$, respectively, were found to elicit HR (Alfano et al., "Analysis of the Role of the Pseudomonas Syringae pv. Syringae HrpZ Harpin in Elicitation of the Hypersensitive Response in Tobacco Using

Functionally Non-polar hrpZ Deletion Mutations, Truncated HrpZ Fragments, and hrmA Mutations," Molecular Microbiology 19:715-728 (1996), which is hereby incorporated by reference). Jin et al., "A Truncated Fragment of Harpin$_{pss}$ Induces Systemic Resistance to Xanthomonas campestris pv. Oryzae in Rice," Physiological and Molecular Plant Pathology 51:243-257 (1997), which is hereby incorporated by reference, reported that a truncated HrpZ$_{Pss}$ with an N-terminal of 137 amino acids elicited a hypersensitive response in tobacco and induced systemic acquired resistance (i.e. SAR) in rice. After digestion with protease, a hypersensitive response active fragment of HrpN$_{Ea}$ was isolated and found to span amino acids 137 to 204 of HrpN$_{Ea}$. It was found that a 98 residue of N-terminal HrpN$_{Ea}$ fragment was the smallest bacterially produced peptide that displayed HR-eliciting activity (Laby, "Molecular Studies on Interactions Between Erwinia Amylovora and its Host and Non-host Plants," Doctoral Thesis in Cornell University (1997), which is hereby incorporated by reference).

A series of HrpN$_{Ea}$ fragments have been generated with His-tag fusion at the N-terminal of the polypeptides and a polypeptide (HrpN$_{Ea}$137180), located at position of 137 to 180 amino acid residue of HrpN$_{Ea}$, was identified to elicit HR activity in tobacco.

Example 7 - Analysis of Secondary Structure of HR Domains

The DNA and primary protein sequence of the HrpN$_{Ea}$137180 show no any homologues among other hypersensitive response elicitors.

Analyses of the secondary structure of the fragment of HrpN$_{Ea}$137180 revealed, with the aid of the computer program Clone Manger5 (Scientific & Educational Software, Durham, NC), that there was a beta-form, a beta-turn, and unordered forms. One typical α-helical segment of residues at 157-170 was found in the HrpN$_{Ea}$137180 polypeptide. To determine the function of this structure, polypeptides with a disrupted α-helical structure were generated and hypersensitive response results were evaluated. As shown in Table 2, a complete alpha-helix unit (H unit), probably with a length greater than 12 amino acid residues, is need for hypersensitive response activity.

Table 2 - Effect of Alpha-helix Structure

Fragment name	Amino acid	HR*	Structure	Source
$HrpN_{Ea}137180$	137-180 (44) pI= 3.10	+ <5 µg/ml	Complete H	E.coli expressed peptide
$HrpN_{Ea}137166$	137-166 (30) pI = 3.29	-	disrupted H	Synthesized peptide
$HrpN_{Ea}76168$	76-168 pI = 3.39	-	disrupted H	E.coli expressed peptide

The α-helical unit plays an important role in hypersensitive response activity; however, it was found that an α-helix unit alone did not achieve HR (Table 3).

Therefore, hypersensitive response eliciting domains contain more than one structure unit. Besides the core α-helical unit, there is an acidic unit that has no typical secondary structure feature but is rich in acidic amino acids. This relaxed structure, having a sheet and random turn, is designated as an acidic unit (A unit).

Although the acidic unit is important in achieving a hypersensitive response, it alone, like the α-helical unit alone, did not elicit a hypersensitive response.

A synthetic polypeptide, $HrpN_{Ea}140176$, that included both A and H structure, spanning amino acids 140 to 176 of $HrpN_{Ea}$, gave full activity of HR. Sequence analysis by major search engines revealed no global primary sequence similarity in the databases to $HrpN_{Ea}140176$, even among the harpin protein families.

Table 3 - Effect of Acidic Unit on Hypersensitive Response (HR) Activity

Fragment name	Amino acid	HR*	Structure (A or H)**	Source
$HrpN_{Ea}140176$	140-176 (37) pI=3.17	+ <5 µg/ml	A + H	Synthesized peptide
$HrpN_{Ea}157170$	157-170 (14) pI = 6.94	-	H	Synthesized peptide
$HrpN_{Ea}137156$	137-156 (20) pI = 2.67	-	A	Synthesized peptide

<u>Example 8</u> - Hypersensitive Response Domain Structure of $HrpN_{Ea}$

Four α-helical regions with at least 12 amino acid residues were found in $HrpN_{Ea}$ based on computer analysis with the program Clone Manager 5 (Scientific & Educational Software, Durham, NC), which predicts the secondary structure of protein from the primary sequence by the method of Garnier-Osguthorpe-Robson.

It is believed that a hypersensitive response domain includes two structural units, the α-helix (H) and the acidic unit (A). Another hypersensitive response domain, spanning amino acids 43 to 70 in HrpNEa, was found. A minimal sequence of 12 to 14 AA residues of both the H and A units is believed to be needed. The chemically synthesized polypeptide of $HrpN_{Ea}$4370 gave full HR activity in tobacco. Thus, a second HR domain has been discovered based on purely secondary structure analysis and prediction.

To further test the hypothesis that the A and H units are needed to achieve a hypersensitive response, an approach of unit exchange (i.e. swapping an acidic unit from one HR domain to another HR domain) was designed. A polypeptide of $HrpN_{Ea}$Dswap, which consisted of the acidic unit of a hypersensitive response domain ($HrpN_{Ea}$140176), spanning amino acids 136 to 156 of $HrpN_{Ea}$, and the α-helical unit of another hypersensitive response domain ($HrpN_{Ea}$4370), spanning amino acids 57 to 70 of $HrpN_{Ea}$, was chemically synthesized. This polypeptide swapped two structural units of A and H between two hypersensitive response domains of $HrpN_{Ea}$4370 and $HrpN_{Ea}$140176. The $HrpN_{Ea}$Dswap gave a hypersensitive response activity in tobacco (Table 4). This result shows that the structural characteristic of an HR domain determines its activity, and structural analysis can be used to determine hypersensitive response activity.

Table 4 - Two Structural Units Determine Hypersensitive Response Activity

Fragment name	Amino acid	HR	Structure Type	Source
$HrpN_{Ea}$4370	43-70 (28) pI= 3.09	+ <5 μg/ml	A + H	Synthesized peptide Partial soluble
$HrpN_{Ea}$Dswap	HrpN136156 (A)+ HrpN5770 (H) pI=2.67	<20 μg/ml	A unit from $HrpN_{Ea}$140176 + H unit from $HrpN_{Ea}$4370	Synthesized peptide Partial soluble

Example 9 - Prediction of Hypersensitive Response Domains Among Proteins in Harpin Family

The secondary structure which indicates the presence of a hypersensitive response domain in HrpNEa was used to identify other harpin proteins, including proteins classified as different subfamilies. Structural prediction of a hypersensitive response domain among harpin proteins was carried according to following criteria:

1. There are two structural units in a hypersensitive response domain, including:
   a. A stable α-helix unit with 12 or more amino acids in length and
   b. An hydrophilic, acidic unit with 12 or more amino acids in length which could be a beta-form, a beta-turn, and unordered forms.
2. The pI of a hypersensitive response domain should be acidic and, in general, below 5.
3. The minimal size of an HR domain is from about 28 to 40 AA residues.

Putative HR domains have been identified to fit the criteria by computer analysis among harpin protein family (Table 5).

Table 5 - Predication of Hypersensitive Response Domains Among Harpin Proteins

HR domain	Isolated Source	Predicted region*	pI	Structure
$HrpN_{Ea}$-1	*E. amylovora*	43-70	3.09	A+H
$HrpN_{Ea}$-2	*E. amylovora*	140-176	3.17	A+H
$HrpN_{Ech}$-1	*E. chrysanthemi*	78-118	5.25	A+H
$HrpN_{Ech}$-2	*E. chrysanthemi*	256-295	4.62	A+H
$HrpN_{Ecc}$-1	*E. carotovora*	25-63	4.06	A+H
$HrpN_{Ecc}$-2	*E. carotovora*	101-140	3.00	A+H
$HrpW_{Pss}$-1	*P. syringae*	52-96	4.32	A+H
$HrpW_{Ea}$-1	*E. amylovora*	10-59	4.53	A+H
$HrpZ_{Pss}$-1	*P. syringae*	97-132	3.68	A+H
$HrpZ_{Pss}$-2	*P. syringae*	153-189	3.67	A+H
$HrpZ_{Pss}$-3	*P. syringae*	271-308	3.95	A+H
$PopA1_{Rs}$-1	*R.solanacearum*	92-125	3.75	A+H
$PopA1_{Rs}$-2	*R.solanacearum*	206-260	3.62	A+H

*Amino acid residue position

Example 10 - Hypersensitive Response Activity of Select Synthesized Polypeptides

Polypeptides were produced by expression in either *E. coli* or by chemical synthesis. Based on prediction of solubility and stability of a particular peptide, in some cases, a broader region of AA residues in addition to the essential units were also synthesized to increase solubility of the peptides. The identification of HR domains among four subfamilies of harpin protein demonstrated this (Table 6).

Table 6 - Hypersensitive Response Activity of Select Synthesized Polypeptides

HR domain	Isolated Source	Synthesized region	pI	Source	HR activity
$HrpN_{Ea}$-1	*E. amylovora*	43-70	3.09	Chemical Synthesized	+ < 5 μg/ml
$HrpN_{Ea}$-2	*E. amylovora*	140-176	3.17	Chemical Synthesized	+ < 5 μg/ml
$HrpW_{Ea}$-2	*E. amylovora*	10-59	4.53	E.coli expressed	+ < 5 μg/ml
$HrpZ_{Pss}$-1	*P. syringae*	97-132	3.68	Chemical Synthesized	+ < 20 μg/ml
$HrpZ_{Pss}$-1	*P. syringae*	153-189	3.69	E.coli expressed	+ < 5 μg/ml
$PopA1_{Rs}$-1	*R. solanacearum*	92-125	3.75	Chemical Synthesized	+ < 5 μg/ml
$PopA1_{Rs}$-2	*R. solanacearum*	206-260	3.62	E.coli expressed	+ < 5 μg/ml

<u>Example 11</u> - Construction of Hypersensitive Response Domains in a Protein Expression Cassette

Polypeptides with a harpin protein hypersensitive response domain were expressed in *E. coli*. PCR was used to amplify desired areas of genes encoding harpin proteins and cloned into an expression vector, e.g. pET28a. A pair of PCR primers with unique flanking sequences were designed to create a universal expression cassette, as shown in Figure 1, for expression of a fragment of harpin protein. Each amplified DNA fragment has a protein translation start codon of ATG in a restriction enzyme Nde I site which might add an extra amino acid of methionine into a polypeptide. Each amplified DNA fragment has a protein translation stop codon of TAA. Each amplified fragment contained two restriction enzyme sites of EcoR V and Sma I, which gave 4 extra in-frame amino acids expressed as Pro-Gly at the N-terminal and Asp-Ile at the C-terminal, respectively. Those two sites are essential to allow two or more expression cassettes to be linked in a specific order and in frame with a minimum number of amino acids being introduced. Cassette A was first digested by EcoR V, ligated to cassette B, and digested with Sma I to produce a new expression cassette C which coupled the two fragments together with two extra amino acids (i.e. Asp-Gly), which are common amino acids in hypersensitive response domains. The newly formed cassette C still contained the same 5' and 3' flanking sequences as original cassettes A and B and maintained the ability to be

coupled by another cassette. Bgl II and Bam HI sites in the cassette permit the cassette to be linked in frame into a cancatomer with a correct orientation. The strategy is that digestion of DNA with Bgl II and Bam HI results in compatible ends that would be ligated with each other but could not be cut by either enzymes after ligation. For example, a DNA fragment encoding a hypersensitive response domain in a cassette could be digested by restrictions enzymes of Bgl II and Bam H1 separately, digested DNA fragments could be ligated in a ligation solution also including both Bgl II and Bam HI enzymes, any ligated ends with Bgl II or Bam HI sites could be digested by the enzymes, and only those ligated sites between Bgl II and Bam HI could remain.

Example 12 - Building Blocks for Creating Superharpins that have Higher Biological Efficacy

Hypersensitive response domains were identified and isolated from several harpin proteins. With the combination of those HR domains, new polypeptides (i.e. superharpins) that have higher HR potency and have enhanced ability to induce disease resistance, impart insect resistance, enhance growth, and achieve environmental stress tolerance. Superharpins could be one HR domain repeat units (cancatomer), different combinations of HR domains, and/or biologically active domains from other elicitors. Part of the domains from different harpin proteins and other elicitors were constructed into the universal expression cassette as shown on Example 11 and designated as superharpin building blocks. Table 7 lists some superharpin building blocks which were expressed in pET-28a(+) vector with a His-tag sequence at their N-terminal.

Table 7 - Superharpin Building Blocks including pET-28a(+) his-tag Leader Sequence

Domain Sequence	Source	MW (kDa)	#a.a.	pI	Soluble	(Structurally) Heat Stable
A	PopA70-146	10.69	104	6.48	Yes	Yes
(N_N)	HrpNEa40-80	6.754	68	6.78	N/A	N/A
$(N_N)_2$	Dimer of HrpNEa40-80	10.84	111	6.13	N/A	N/A
$(N_N)_3$	Triplemer of HrpNEa40-80	14.93	154	5.63	N/A	N/A
$(N_N)_4$	Tetramer of HrpNEa40-80	19.01	197	4.95	N/A	N/A
(N_C)	HrpNEa140-180	7.224	68	5.01	Yes	Yes
$(N_C)_2$	Dimer of HrpNEa140-180	11.78	111	3.98	Yes	Yes
$(N_C)_3$	Triplemer of HrpNEa140-180	16.34	154	3.72	Yes	Yes
$(N_C)_4$	Tetramer of HrpNEa140-180	20.89	197	3.58	Yes	Yes
$(N_C)_{10}$	Cancatomer (10 repeating units of HrpNEa140-180	48.23	455	3.28	N/A	N/A
$(N_C)_{16}$	Cancatomer (16 repeating units of HrpNEa140-180	75.57	713	3.18	N/A	N/A
W	HrpWEa10-59	7.986	77	6.48	N/A	N/A
Z_N	HrpZ90-150	8.087	78	5.38	Yes	Yes
$Z_{266-308}$	HrpZ266-308	7.029	70	6.40	Yes	Yes
his-tag leader seq.		2.045	19	11.04		

Example 13 - Superharpins with Stacked HR Domains and their Biological Activities

There are numerous polypeptides could be generated with different combinations of HR domains or by stacking HR domains and repeating units in order. Selective combination or stacking of HR domains isolated from harpin proteins or other elicitors can be designed to achieve a targeted disease resistance spectrum. See Table 8 for superharpins prepared by stacking of HR building blocks listed on Table 7. All three listed superharpins (i.e. SH-1, SH-2, SH-3) were constructed into a pET28(a) vector and expressed in *E. coli*. Recombinant proteins were partially purified and quantified by SDS-PAGE with purified Harpin N protein as a quantitative standard.

Table 8 - Properties of Superharpins

Protein	Domain Sequence	MW (kDa)	# a.a.	pI	Soluble	Heat Stable
SH-1	*$W(N_N)_4A(N_C)_4Z_{266\text{-}308}$	54.955	545	3.69	Yes	Yes
SH-2	*$W(N_N)_4Z_N(N_C)_4Z_{266\text{-}308}$	52.341	519	3.54	Yes	Yes
SH-3	*$W(N_N)_4Z_N(N_C)_4Z_{266\text{-}308}A$	60.375	598	3.67	Yes	Yes
HrpNEa	HrpN from E.amylovora	39.697	403	4.42	Yes	Yes

Bioassays for hypersensitive response on tobacco leaves (HR), percentage of TMV reduction on tobacco leaves, and plant growth enhancement with tomato showed that superharpins had higher (up to 2 to 10 fold greater) HR potency compared with HrpN from *E. amylovora*. This also demonstrated that superharpins have better performance on % TMV reduction and plant growth enhancement assay. See Table 9.

Table 9 - Biological Activities of Superharpins

Protein	Domain Sequence	Elicit HR (~μg/ml)	% TMV reduction on tobacco		% Plant Growth Enhancement	
			10 μg/ml	1 μg/ml	10 μg/ml	1 μg/ml
SH-1	$W(N_N)_4A(N_C)_4Z_{266\text{-}308}$	0.66	83	79	7.49	9.83
SH-2	$W(N_N)_4Z_N(N_C)_4Z_{266\text{-}308}$	0.13	84	60	11.05	7.30
SH-3	$W(N_N)_4Z_N(N_C)_4Z_{266\text{-}308}A$	0.15	77	55	11.07	10.00
HrpNEa	HrpN from E.amylovora	1-3	55	10	11.68	N/A

Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

WHAT IS CLAIMED:

1. An isolated hypersensitive response elicitor protein comprising an isolated pair or more of spaced apart domains, each comprising an acidic portion linked to an alpha-helix and capable of eliciting a hypersensitive response in plants.

2. A protein according to claim 1, wherein the protein is recombinant.

3. An isolated nucleic acid molecule encoding a protein according to claim 1.

4. A nucleic acid molecule according to claim 3, wherein each domain is from a different source organism.

5. A nucleic acid molecule according to claim 3, wherein there are 3 or more spaced apart domains.

6. An expression vector containing a nucleic acid molecule according to claim 3 which is heterologous to the expression vector.

7. An expression vector according to claim 6, wherein the nucleic acid molecule is positioned in the expression vector in sense orientation and correct reading frame.

8. A host cell transformed with the nucleic acid molecule according to claim 3.

9. A host cell transformed according to claim 8, wherein the host cell is selected from the group consisting of a plant cell, a eukaryotic cell, and a procaryotic cell.

10. A host cell according to claim 8, wherein the nucleic acid molecule is transformed with an expression system.

11. A transgenic plant transformed with the nucleic acid molecule of claim 3.

12. A transgenic plant according to claim 11, wherein the plant is selected from the group consisting of alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

13. A transgenic plant according to claim 11, wherein the plant is selected from the group consisting of *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

14. A transgenic plant according to claim 11, wherein the plant is a monocot.

15. A transgenic plant according to claim 11, wherein the plant is a dicot.

16. A transgenic plant according to claim 11, wherein each domain is from a different source organism.

17. A transgenic plant according to claim 11, wherein there are 3 or more spaced apart domains.

18. A transgenic plant seed transformed with the nucleic acid molecule of claim 3.

19. A transgenic plant seed according to claim 18, wherein the plant is selected from the group consisting of alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

20. A transgenic plant seed according to claim 18, wherein the plant is selected from the group consisting of *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

21. A transgenic plant seed according to claim 18, wherein the plant is a monocot.

22. A transgenic plant seed according to claim 18, wherein the plant is a dicot.

23. A method of imparting disease resistance to plants comprising:

applying a protein according to claim 1 to a plant or a plant seed under conditions effective to impart disease resistance to the plant or to a plant grown from the plant seed.

24. A method according to claim 23, wherein the protein is applied to a plant.

25. A method according to claim 23, wherein the protein is applied to a plant seed and further comprising:

planting the plant seed under conditions effective to impart disease resistance to a plant grown from the plant seeds.

26. A method of enhancing plant growth comprising:

applying a protein according to claim 1 to a plant or a plant seed under conditions effective to enhance growth of the plants or of a plant grown from the plant seed.

27. A method according to claim 26, wherein the protein is applied to a plant.

28. A method according to claim 26, wherein the protein is applied to a plant seed and further comprising:

planting the plant seeds under conditions effective to enhance growth of a plant grown from the plant seed.

29. A method of controlling insects comprising:

applying a protein according to claim 1 to a plant or a plant seed under conditions effective to control insects.

30. A method according to claim 29, wherein the protein is applied to a plant.

31. A method according to claim 29, wherein the protein is applied to a plant seed and further comprising:

planting the plant seed under conditions effective to grow a plant from the plant seed and to control insects.

32. A method of imparting stress resistance to plants comprising:

applying a protein according to claim 1 to a plant or a plant seed under conditions effective to impart stress resistance to the plant or to a plant grown from the plant seed.

33. A method according to claim 32, wherein the protein is applied to a plant.

34. A method according to claim 32, wherein the protein is applied to a plant seed and further comprising:

planting the plant seed under conditions effective to impart stress resistance to a plant grown from the plant seed.

35. A method of imparting disease resistance to plants comprising:

providing a transgenic plant or transgenic plant seed containing the nucleic acid according to claim 3 and

planting the transgenic plant or transgenic plant seed under conditions effective to impart disease resistance to the plant or to a plant grown from the plant seed.

36. A method according to claim 35, wherein a transgenic plant is provided.

37. A method according to claim 35, wherein a transgenic plant seed is provided.

38. A method of enhancing growth of plants comprising:

providing a transgenic plant or transgenic plant seed containing the nucleic acid according to claim 3 and

planting the transgenic plant or transgenic plant seed under conditions effective to enhance growth of the plant or of a plant grown from the plant seed.

39. A method according to claim 38, wherein a transgenic plant is provided.

40. A method according to claim 38, wherein a transgenic plant seed is provided.

41. A method of controlling insects comprising:

providing a transgenic plant or transgenic plant seed containing the nucleic acid according to claim 3 and

planting the transgenic plant or transgenic plant seed under conditions effective to control insects on the plant or on a plant grown from the plant seed.

42. A method according to claim 41, wherein a transgenic plant is provided.

43. A method according to claim 41, wherein a transgenic plant seed is provided.

44. A method of imparting stress resistance to plants comprising:

providing a transgenic plant or transgenic plant seed containing the nucleic acid according to claim 3 and

planting the transgenic plant or transgenic plant seed under conditions effective to impart stress resistance to the plant or to a plant grown from the plant seed.

45. A method according to claim 44, wherein a transgenic plant is provided.

46. A method according to claim 44, wherein a transgenic plant seed is provided.

47. An isolated hypersensitive response elicitor protein comprising, in isolation, a domain comprising an acid portion linked to an alpha-helix and capable of eliciting a hypersensitive response in plants.

48. A protein according to claim 47, wherein the protein is recombinant.

49. An isolated nucleic acid molecule encoding a protein according to claim 47.

50. An isolated nucleic acid molecule according to claim 49, wherein there are at least 2 domains, each from a different source organism.

51. An isolated nucleic acid molecule according to claim 49, wherein there are 3 or more coupled domains.

52. An expression vector containing a nucleic acid molecule according to claim 49 which is heterologous to the expression vector.

53. An expression vector according to claim 52, wherein the nucleic acid molecule is positioned in the expression vector in sense orientation and correct reading frame.

54. A host cell transformed with the nucleic acid molecule according to claim 49.

55. A host cell transformed according to claim 54, wherein the host cell is selected from the group consisting of a plant cell, a eukaryotic cell, and a prokaryotic cell.

56. A host cell according to claim 54, wherein the nucleic acid molecule is transformed with an expression system.

57. A transgenic plant transformed with the nucleic acid molecule of claim 49.

58. A transgenic plant according to claim 57, wherein the plant is selected from the group consisting of alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean pea, chicory, lettuce, endive,

cabbage, brussel sprout, beet, parsnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

59. A transgenic plant according to claim 57, wherein the plant is selected from the group consisting of *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

60. A transgenic plant according to claim 57, wherein the plant is a monocot.

61. A transgenic plant according to claim 57, wherein the plant is a dicot.

62. A transgenic plant according to claim 57, wherein there are at least 2 coupled domains, each from a different source organism.

63. A transgenic plant according to claim 57, wherein there are 3 or more coupled domains.

64. A transgenic plant seed transformed with the nucleic acid molecule of claim 49.

65. A transgenic plant seed according to claim 64, wherein the plant is selected from the group consisting of alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

66. A transgenic plant seed according to claim 64, wherein the plant is selected from the group consisting of *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

67. A transgenic plant seed according to claim 64, wherein the plant is a monocot.

68. A transgenic plant seed according to claim 64, wherein the plant is a dicot.

69. A method of imparting disease resistance to plants comprising:

applying a protein according to claim 47 to a plant or a plant seed under conditions effective to impart disease resistance to the plant or to a plant grown from the plant seed.

70. A method according to claim 69, wherein the protein is applied to a plant.

71. A method according to claim 69, wherein the protein is applied to a plant seed and further comprising:

planting the plant seed under conditions effective to impart disease resistance to a plant grown from the plant seed.

72. A method of enhancing plant growth comprising:

applying a protein according to claim 47 to a plant or a plant seed under conditions effective to enhance growth of the plant or of a plant grown from the plant seed.

73. A method according to claim 72, wherein the protein is applied to a plant.

74. A method according to claim 72, wherein the protein is applied to a plant seed and further comprising:

planting the plant seed under conditions effective to enhance growth of a plant grown from the plant seed.

75. A method of controlling insects comprising:

applying a protein according to claim 47 to a plant or a plant seed under conditions effective to control insects.

76. A method according to claim 75, wherein the protein is applied to a plant.

77. A method according to claim 75, wherein the protein is applied to a plant seed and further comprising:

planting the plant seed under conditions effective to grow a plant from the plant seed and to control insects.

78. A method of imparting stress resistance to plants comprising:

applying a protein according to claim 47 to a plant or a plant seed under conditions effective to impart stress resistance to the plant or to a plant grown from the plant seed.

79. A method according to claim 78, wherein the protein is applied to a plant.

80. A method according to claim 78, wherein the protein is applied to a plant seed and further comprising:

planting the plant seed under conditions effective to impart stress resistance to a plant grown from the plant seed.

81. A method of imparting disease resistance to plants comprising:

providing a transgenic plant or transgenic plant seed containing the nucleic acid according to claim 49 and

planting the transgenic plant or transgenic plant seed under conditions effective to impart disease resistance to the plant or to a plant grown from the plant seed.

82. A method according to claim 81, wherein a transgenic plant is provided.

83. A method according to claim 81, wherein a transgenic plant seed is provided.

84. A method of enhancing growth of plants comprising:

providing a transgenic plant or transgenic plant seed containing the nucleic acid according to claim 49 and

planting the transgenic plant or transgenic plant seed under conditions effective to enhance growth of the plant or of a plant grown from the plant seed.

85. A method according to claim 84, wherein a transgenic plant is provided.

86. A method according to claim 84, wherein a transgenic plant seed is provided.

87. A method of controlling insects comprising:

providing a transgenic plant or transgenic plant seed containing the nucleic acid according to claim 49 and

planting the transgenic plant or transgenic plant seed under conditions effective to control insects on the plant or on a plant grown from the plant seed.

88. A method according to claim 87, wherein a transgenic plant is provided.

89. A method according to claim 87, wherein a transgenic plant seed is provided.

90. A method of imparting stress resistance to plants comprising:

providing a transgenic plant or transgenic plant seed containing the nucleic acid according to claim 49 and

planting the transgenic plant or transgenic plant seed under conditions effective to impart stress resistance to the plant or to a plant grown from the plant seed.

91. A method according to claim 90, wherein a transgenic plant is provided.

92. A method according to claim 90, wherein a transgenic plant seed is provided.

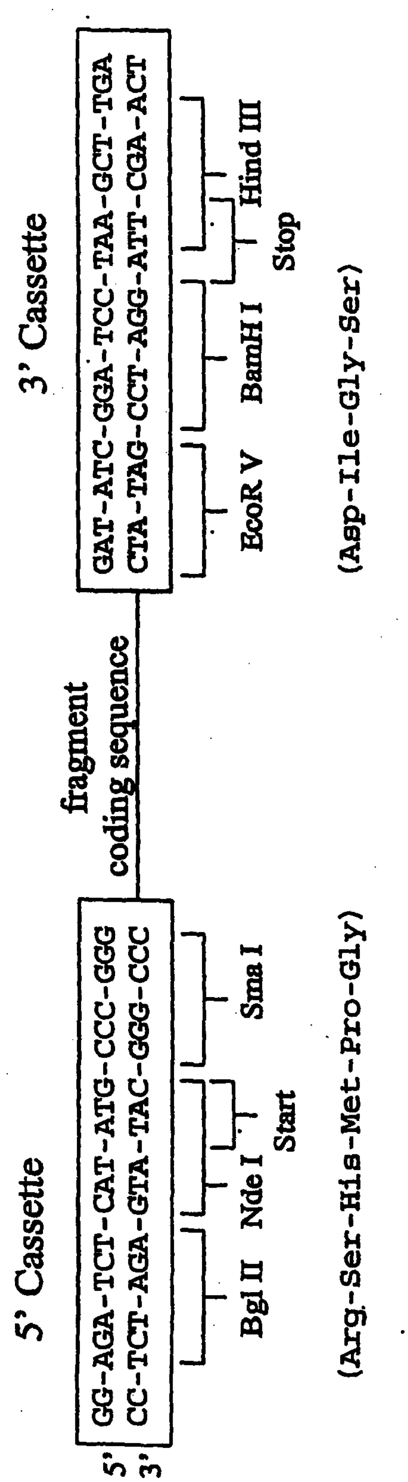

5' Cassette
5'
3'
GG-AGA-TCT-CAT-ATG-CCC-GGG
CC-TCT-AGA-GTA-TAC-GGG-CCC
Bgl II
Nde I
Start
Sma I
(Arg-Ser-His-Met-Pro-Gly)
fragment
coding sequence
3' Cassette
GAT-ATC-GGA-TCC-TAA-GCT-TGA
CTA-TAG-CCT-AGG-ATT-CGA-ACT
EcoR V
BamH I
Stop
Hind III
(Asp-Ile-Gly-Ser)

Figure 1

SEQUENCE LISTING

<110> Eden Bioscience Corporation

<120> HYPERSENSITIVE RESPONSE ELICITING DOMAINS AND USE THEREOF

<130> 21829/82

<140>
<141>

<150> 60/212,211
<151> 2000-06-16

<160> 18

<170> PatentIn Ver. 2.1

<210> 1
<211> 338
<212> PRT
<213> Erwinia chrysanthemi

<400> 1
```
Met Gln Ile Thr Ile Lys Ala His Ile Gly Gly Asp Leu Gly Val Ser
  1               5                  10                  15

Gly Leu Gly Ala Gln Gly Leu Lys Gly Leu Asn Ser Ala Ala Ser Ser
             20                  25                  30

Leu Gly Ser Ser Val Asp Lys Leu Ser Ser Thr Ile Asp Lys Leu Thr
         35                  40                  45

Ser Ala Leu Thr Ser Met Met Phe Gly Gly Ala Leu Ala Gln Gly Leu
     50                  55                  60

Gly Ala Ser Ser Lys Gly Leu Gly Met Ser Asn Gln Leu Gly Gln Ser
 65                  70                  75                  80

Phe Gly Asn Gly Ala Gln Gly Ala Ser Asn Leu Leu Ser Val Pro Lys
                 85                  90                  95

Ser Gly Gly Asp Ala Leu Ser Lys M t Ph  Asp Lys Ala Leu Asp Asp
            100                 105                 110

Leu Leu Gly His Asp Thr Val Thr Lys Leu Thr Asn Gln Ser Asn Gln
        115                 120                 125
```

Leu Ala Asn Ser Met Leu Asn Ala Ser Gln Met Thr Gln Gly Asn Met
    130   135   140

Asn Ala Phe Gly Ser Gly Val Asn Asn Ala Leu Ser Ser Ile Leu Gly
145   150   155   160

Asn Gly Leu Gly Gln Ser Met Ser Gly Phe Ser Gln Pro Ser Leu Gly
                165   170   175

Ala Gly Gly Leu Gln Gly Leu Ser Gly Ala Gly Ala Phe Asn Gln Leu
            180   185   190

Gly Asn Ala Ile Gly Met Gly Val Gly Gln Asn Ala Ala Leu Ser Ala
        195   200   205

Leu Ser Asn Val Ser Thr His Val Asp Gly Asn Asn Arg His Phe Val
    210   215   220

Asp Lys Glu Asp Arg Gly Met Ala Lys Glu Ile Gly Gln Phe Met Asp
225   230   235   240

Gln Tyr Pro Glu Ile Phe Gly Lys Pro Glu Tyr Gln Lys Asp Gly Trp
                245   250   255

Ser Ser Pro Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser Lys
            260   265   270

Pro Asp Asp Asp Gly Met Thr Gly Ala Ser Met Asp Lys Phe Arg Gln
        275   280   285

Ala Met Gly Met Ile Lys Ser Ala Val Ala Gly Asp Thr Gly Asn Thr
    290   295   300

Asn Leu Asn Leu Arg Gly Ala Gly Gly Ala Ser Leu Gly Ile Asp Ala
305   310   315   320

Ala Val Val Gly Asp Lys Ile Ala Asn Met Ser Leu Gly Lys Leu Ala
                325   330   335

Asn Ala

<210> 2
<211> 2141
<212> DNA
<213> Erwinia chrysanthemi

<400> 2
cgattttacc cgggtgaacg tgctatgacc gacagcatca cggtattcga caccgttacg 60
gcgtttatgg ccgcgatgaa ccggcatcag gcggcgcgct ggtcgccgca atccggcgtc 120
gatctggtat ttcagtttgg ggacaccggg cgtgaactca tgatgcagat tcagccgggg 180
cagcaatatc ccggcatgtt gcgcacgctg ctcgctcgtc gttatcagca ggcggcagag 240
tgcgatggct gccatctgtg cctgaacggc agcgatgtat tgatcctctg gtggccgctg 300
ccgtcggatc ccggcagtta tccgcaggtg atcgaacgtt tgtttgaact ggcgggaatg 360
acgttgccgt cgctatccat agcaccgacg gcgcgtccgc agacagggaa cggacgcgcc 420
cgatcattaa gataaaggcg gcttttttta ttgcaaaacg gtaacggtga ggaaccgttt 480
caccgtcggc gtcactcagt aacaagtatc catcatgatg cctacatcgg gatcggcgtg 540
ggcatccgtt gcagatactt ttgcgaacac ctgacatgaa tgaggaaacg aaattatgca 600
aattacgatc aaagcgcaca tcggcggtga tttgggcgtc tccggtctgg ggctgggtgc 660
tcagggactg aaaggactga attccgcggc ttcatcgctg ggttccagcg tggataaact 720
gagcagcacc atcgataagt tgacctccgc gctgacttcg atgatgtttg gcggcgcgct 780
ggcgcagggg ctgggcgcca gctcgaaggg gctggggatg agcaatcaac tgggccagtc 840
tttcggcaat ggcgcgcagg gtgcgagcaa cctgctatcc gtaccgaaat ccggcggcga 900
tgcgttgtca aaaatgtttg ataaagcgct ggacgatctg ctgggtcatg acaccgtgac 960
caagctgact aaccagagca accaactggc taattcaatg ctgaacgcca gccagatgac 1020
ccagggtaat atgaatgcgt tcggcagcgg tgtgaacaac gcactgtcgt ccattctcgg 1080
caacggtctc ggccagtcga tgagtggctt ctctcagcct tctctggggg caggcggctt 1140
gcagggcctg agcggcgcgg gtgcattcaa ccagttgggt aatgccatcg gcatgggcgt 1200
ggggcagaat gctgcgctga gtgcgttgag taacgtcagc acccacgtag acggtaacaa 1260
ccgccacttt gtagataaag aagatcgcgg catggcgaaa gagatcggcc agtttatgga 1320
tcagtatccg gaaatattcg gtaaaccgga ataccagaaa gatggctgga gttcgccgaa 1380
gacggacgac aaatcctggg ctaaagcgct gagtaaaccg gatgatgacg gtatgaccgg 1440
cgccagcatg gacaaattcc gtcaggcgat gggtatgatc aaaagcgcgg tggcgggtga 1500
taccggcaat accaacctga acctgcgtgg cgcgggcggt gcatcgctgg gtatcgatgc 1560
ggctgtcgtc ggcgataaaa tagccaacat gtcgctgggt aagctggcca acgcctgata 1620
atctgtgctg gcctgataaa gcggaaacga aaaaagagac ggggaagcct gtctcttttc 1680
ttattatgcg gtttatgcgg ttacctggac cggttaatca tcgtcatcga tctggtacaa 1740
acgcacattt tcccgttcat tcgcgtcgtt acgcgccaca atcgcgatgg catcttcctc 1800
gtcgctcaga ttgcgcggct gatggggaac gccgggtgga atatagagaa actcgccggc 1860
cagatggaga cacgtctgcg ataaatctgt gccgtaacgt gtttctatcc gcccctttag 1920
cagatagatt gcggtttcgt aatcaacatg gtaatgcggt tccgcctgtg cgccggccgg 1980
gatcaccaca atattcatag aaagctgtct tgcacctacc gtatcgcggg agataccgac 2040
aaaatagggc agtttttgcg tggtatccgt ggggtgttcc ggcctgacaa tcttgagttg 2100
gttcgtcatc atctttctcc atctgggcga cctgatcggt t 2141

<210> 3
<211> 403
<212> PRT
<213> Erwinia amylovora

<400> 3
Met Ser Leu Asn Thr Ser Gly Leu Gly Ala Ser Thr Met Gln Ile Ser
1 5 10 15

Ile Gly Gly Ala Gly Gly Asn Asn Gly Leu Leu Gly Thr Ser Arg Gln
20 25 30

Asn Ala Gly Leu Gly Gly Asn Ser Ala Leu Gly Leu Gly Gly Gly Asn
35 40 45

Gln Asn Asp Thr Val Asn Gln Leu Ala Gly Leu Leu Thr Gly Met Met
50 55 60

Met Met Met Ser Met Met Gly Gly Gly Gly Leu Met Gly Gly Gly Leu
65 70 75 80

Gly Gly Gly Leu Gly Asn Gly Leu Gly Gly Ser Gly Gly Leu Gly Glu
85 90 95

Gly Leu Ser Asn Ala Leu Asn Asp Met Leu Gly Gly Ser Leu Asn Thr
100 105 110

Leu Gly Ser Lys Gly Gly Asn Asn Thr Thr Ser Thr Thr Asn Ser Pro
115 120 125

Leu Asp Gln Ala Leu Gly Ile Asn Ser Thr Ser Gln Asn Asp Asp Ser
130 135 140

Thr Ser Gly Thr Asp Ser Thr Ser Asp Ser Ser Asp Pro Met Gln Gln
145 150 155 160

Leu Leu Lys Met Phe Ser Glu Ile Met Gln Ser Leu Phe Gly Asp Gly
165 170 175

Gln Asp Gly Thr Gln Gly Ser Ser Ser Gly Gly Lys Gln Pro Thr Glu
180 185 190

Gly Glu Gln Asn Ala Tyr Lys Lys Gly Val Thr Asp Ala Leu Ser Gly
195 200 205

Leu Met Gly Asn Gly Leu Ser Gln Leu Leu Gly Asn Gly Gly Leu Gly
210 215 220

Gly Gly Gln Gly Gly Asn Ala Gly Thr Gly Leu Asp Gly Ser Ser Leu
225 230 235 240

Gly Gly Lys Gly Leu Gln Asn Leu Ser Gly Pro Val Asp Tyr Gln Gln
245 250 255

Leu Gly Asn Ala Val Gly Thr Gly Ile Gly Met Lys Ala Gly Ile Gln
260 265 270

Ala Leu Asn Asp Ile Gly Thr His Arg His Ser Ser Thr Arg Ser Phe
275 280 285

Val Asn Lys Gly Asp Arg Ala Met Ala Lys Glu Ile Gly Gln Phe Met
290 295 300

Asp Gln Tyr Pro Glu Val Phe Gly Lys Pro Gln Tyr Gln Lys Gly Pro
305 310 315 320

Gly Gln Glu Val Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser
325 330 335

Lys Pro Asp Asp Asp Gly Met Thr Pro Ala Ser Met Glu Gln Phe Asn
340 345 350

Lys Ala Lys Gly Met Ile Lys Arg Pro Met Ala Gly Asp Thr Gly Asn
355 360 365

Gly Asn Leu Gln Ala Arg Gly Ala Gly Gly Ser Ser Leu Gly Ile Asp
370 375 380

Ala Met Met Ala Gly Asp Ala Ile Asn Asn Met Ala Leu Gly Lys Leu
385 390 395 400

Gly Ala Ala

<210> 4
<211> 1288
<212> DNA
<213> Erwinia amylovora

<400> 4
aagcttcggc atggcacgtt tgaccgttgg gtcggcaggg tacgtttgaa ttattcataa 60
gaggaatacg ttatgagtct gaatacaagt gggctgggag cgtcaacgat gcaaatttct 120
atcggcggtg cgggcggaaa taacgggttg ctgggtacca gtcgccagaa tgctgggttg 180
ggtggcaatt ctgcactggg gctgggcggc ggtaatcaaa atgataccgt caatcagctg 240
gctggcttac tcaccggcat gatgatgatg atgagcatga tgggcggtgg tgggctgatg 300
ggcggtggct taggcggtgg cttaggtaat ggcttgggtg gctcaggtgg cctgggcgaa 360
ggactgtcga acgcgctgaa cgatatgtta ggcggttcgc tgaacacgct gggctcgaaa 420
ggcggcaaca ataccacttc aacaacaaat tccccgctgg accaggcgct gggtattaac 480
tcaacgtccc aaaacgacga ttccacctcc ggcacagatt ccacctcaga ctccagcgac 540
ccgatgcagc agctgctgaa gatgttcagc gagataatgc aaagcctgtt tggtgatggg 600
caagatggca cccagggcag ttcctctggg ggcaagcagc cgaccgaagg cgagcagaac 660
gcctataaaa aaggagtcac tgatgcgctg tcgggcctga tgggtaatgg tctgagccag 720
ctccttggca acgggggact gggaggtggt cagggcggta atgctggcac gggtcttgac 780

ggttcgtcgc tgggcggcaa agggctgcaa aacctgagcg ggccggtgga ctaccagcag 840
ttaggtaacg ccgtgggtac cggtatcggt atgaaagcgg gcattcaggc gctgaatgat 900
atcggtacgc acaggcacag ttcaaccccgt tctttcgtca ataaaggcga tcgggcgatg 960
gcgaaggaaa tcggtcagtt catggaccag tatcctgagg tgtttggcaa gccgcagtac 1020
cagaaaggcc cgggtcagga ggtgaaaacc gatgacaaat catgggcaaa agcactgagc 1080
aagccagatg acgacggaat gacaccagcc agtatggagc agttcaacaa agccaagggc 1140
atgatcaaaa ggcccatggc gggtgatacc ggcaacggca acctgcaggc acgcggtgcc 1200
ggtggttctt cgctgggtat tgatgccatg atggccggtg atgccattaa caatatggca 1260
cttggcaagc tgggcgcggc ttaagctt 1288

<210> 5
<211> 1344
<212> DNA
<213> Erwinia amylovora

<400> 5
atgtcaattc ttacgcttaa caacaatacc tcgtcctcgc cgggtctgtt ccagtccggg 60
ggggacaacg ggcttggtgg tcataatgca aattctgcgt tggggcaaca acccatcgat 120
cggcaaacca ttgagcaaat ggctcaatta ttggcggaac tgttaaagtc actgctatcg 180
ccacaatcag gtaatgcggc aaccggagcc ggtggcaatg accagactac aggagttggt 240
aacgctggcg gcctgaacgg acgaaaaggc acagcaggaa ccactccgca gtctgacagt 300
cagaacatgc tgagtgagat gggcaacaac gggctggatc aggccatcac gcccgatggc 360
cagggcggcg ggcagatcgg cgataatcct ttactgaaag ccatgctgaa gcttattgca 420
cgcatgatgg acggccaaag cgatcagttt ggccaacctg gtacgggcaa caacagtgcc 480
tcttccggta cttcttcatc tggcggttcc cctttttaacg atctatcagg ggggaaggcc 540
ccttccggca actccccttc cggcaactac tctcccgtca gtaccttctc acccccatcc 600
acgccaacgt cccctacctc accgcttgat ttcccttctt ctcccaccaa agcagccggg 660
ggcagcacgc cggtaaccga tcatcctgac cctgttggta gcgcgggcat cggggccgga 720
aattcggtgg ccttcaccag cgccggcgct aatcagacgg tgctgcatga caccattacc 780
gtgaaagcgg gtcaggtgtt tgatggcaaa ggacaaacct tcaccgccgg ttcagaatta 840
ggcgatggcg gccagtctga aaaccagaaa ccgctgttta tactggaaga cggtgccagc 900
ctgaaaaacg tcaccatggg cgacgacggg gcggatggta ttcatcttta cggtgatgcc 960
aaaatagaca atctgcacgt caccaacgtg ggtgaggacg cgattaccgt taagccaaac 1020
agcgcgggca aaaaatccca cgttgaaatc actaacagtt ccttcgagca cgcctctgac 1080
aagatcctgc agctgaatgc cgatactaac ctgagcgttg acaacgtgaa ggccaaagac 1140
tttggtactt ttgtacgcac taacggcggt caacagggta actgggatct gaatctgagc 1200
catatcagcg cagaagacgg taagttctcg ttcgttaaaa gcgatagcga ggggctaaac 1260
gtcaatacca gtgatatctc actgggtgat gttgaaaacc actacaaagt gccgatgtcc 1320
gccaacctga aggtggctga atga 1344

<210> 6
<211> 447
<212> PRT
<213> Erwinia amylovora

<400> 6

Met Ser Ile Leu Thr Leu Asn Asn Asn Thr Ser Ser Ser Pro Gly Leu
1 5 10 15

Phe Gln Ser Gly Gly Asp Asn Gly Leu Gly Gly His Asn Ala Asn Ser
20 25 30

Ala Leu Gly Gln Gln Pro Ile Asp Arg Gln Thr Ile Glu Gln Met Ala
35 40 45

Gln Leu Leu Ala Glu Leu Leu Lys Ser Leu Leu Ser Pro Gln Ser Gly
50 55 60

Asn Ala Ala Thr Gly Ala Gly Gly Asn Asp Gln Thr Thr Gly Val Gly
65 70 75 80

Asn Ala Gly Gly Leu Asn Gly Arg Lys Gly Thr Ala Gly Thr Thr Pro
85 90 95

Gln Ser Asp Ser Gln Asn Met Leu Ser Glu Met Gly Asn Asn Gly Leu
100 105 110

Asp Gln Ala Ile Thr Pro Asp Gly Gln Gly Gly Gly Gln Ile Gly Asp
115 120 125

Asn Pro Leu Leu Lys Ala Met Leu Lys Leu Ile Ala Arg Met Met Asp
130 135 140

Gly Gln Ser Asp Gln Phe Gly Gln Pro Gly Thr Gly Asn Asn Ser Ala
145 150 155 160

Ser Ser Gly Thr Ser Ser Ser Gly Gly Ser Pro Phe Asn Asp Leu Ser
165 170 175

Gly Gly Lys Ala Pro Ser Gly Asn Ser Pro Ser Gly Asn Tyr Ser Pro
180 185 190

Val Ser Thr Phe Ser Pro Pro Ser Thr Pro Thr Ser Pro Thr Ser Pro
195 200 205

Leu Asp Phe Pro Ser Ser Pro Thr Lys Ala Ala Gly Gly Ser Thr Pro
210 215 220

Val Thr Asp His Pro Asp Pro Val Gly Ser Ala Gly Ile Gly Ala Gly
225 230 235 240

Asn Ser Val Ala Phe Thr Ser Ala Gly Ala Asn Gln Thr Val Leu His
245 250 255

Asp Thr Ile Thr Val Lys Ala Gly Gln Val Phe Asp Gly Lys Gly Gln
260 265 270

Thr Phe Thr Ala Gly Ser Glu Leu Gly Asp Gly Gly Gln Ser Glu Asn
275 280 285

Gln Lys Pro Leu Phe Ile Leu Glu Asp Gly Ala Ser Leu Lys Asn Val
290 295 300

Thr Met Gly Asp Asp Gly Ala Asp Gly Ile His Leu Tyr Gly Asp Ala
305 310 315 320

Lys Ile Asp Asn Leu His Val Thr Asn Val Gly Glu Asp Ala Ile Thr
325 330 335

Val Lys Pro Asn Ser Ala Gly Lys Lys Ser His Val Glu Ile Thr Asn
340 345 350

Ser Ser Phe Glu His Ala Ser Asp Lys Ile Leu Gln Leu Asn Ala Asp
355 360 365

Thr Asn Leu Ser Val Asp Asn Val Lys Ala Lys Asp Phe Gly Thr Phe
370 375 380

Val Arg Thr Asn Gly Gly Gln Gln Gly Asn Trp Asp Leu Asn Leu Ser
385 390 395 400

His Ile Ser Ala Glu Asp Gly Lys Phe Ser Phe Val Lys Ser Asp Ser
405 410 415

Glu Gly Leu Asn Val Asn Thr Ser Asp Ile Ser Leu Gly Asp Val Glu
420 425 430

Asn His Tyr Lys Val Pro Met Ser Ala Asn Leu Lys Val Ala Glu
435 440 445

<210> 7
<211> 5517
<212> DNA
<213> Erwinia amylovora

<400> 7
atggaattaa aatcactggg aactgaacac aaggcggcag tacacacagc ggcgcacaac 60
cctgtggggc atggtgttgc cttacagcag ggcagcagca gcagcagccc gcaaaatgcc 120
gctgcatcat tggcggcaga aggcaaaaat cgtgggaaaa tgccgagaat tcaccagcca 180
tctactgcgg ctgatggtat cagcgctgct caccagcaaa agaaatcctt cagtctcagg 240
ggctgtttgg ggacgaaaaa attttccaga tcggcaccgc agggccagcc aggtaccacc 300

cacagcaaag gggcaacatt gcgcgatctg ctggcgcggg acgacggcga aacgcagcat 360
gaggcggccg cgccagatgc ggcgcgtttg acccgttcgg gcggcgtcaa acgccgcaat 420
atggacgaca tggccgggcg gccaatggtg aaaggtggca gcggcgaaga taaggtacca 480
acgcagcaaa aacggcatca gctgaacaat tttggccaga tgcgccaaac gatgttgagc 540
aaaatggctc acccggcttc agccaacgcc ggcgatcgcc tgcagcattc accgccgcac 600
atcccgggta gccaccacga aatcaaggaa gaaccggttg gctccaccag caaggcaaca 660
acggcccacg cagacagagt ggaaatcgct caggaagatg acgacagcga attccagcaa 720
ctgcatcaac agcggctggc gcgcgaacgg gaaaatccac cgcagccgcc caaactcggc 780
gttgccacac cgattagcgc caggtttcag cccaaactga ctgcggttgc ggaaagcgtc 840
cttgagggga cagataccac gcagtcaccc cttaagccgc aatcaatgct gaaaggaagt 900
ggagccgggg taacgccgct ggcggtaacg ctggataaag gcaagttgca gctggcaccg 960
gataatccac ccgcgctcaa tacgttgttg aagcagacat tgggtaaaga cacccagcac 1020
tatctggcgc accatgccag cagcgacggt agccagcatc tgctgctgga caacaaaggc 1080
cacctgtttg atatcaaaag caccgccacc agctatagcg tgctgcacaa cagccacccc 1140
ggtgagataa agggcaagct ggcgcaggcg ggtactggct ccgtcagcgt agacggtaaa 1200
agcggcaaga tctcgctggg gagcggtacg caaagtcaca acaaaacaat gctaagccaa 1260
ccgggggaag cgcaccgttc cttattaacc ggcatttggc agcatcctgc tggcgcagcg 1320
cggccgcagg gcgagtcaat ccgcctgcat gacgacaaaa ttcatatcct gcatccggag 1380
ctgggcgtat ggcaatctgc ggataaagat acccacagcc agctgtctcg ccaggcagac 1440
ggtaagctct atgcgctgaa agacaaccgt accctgcaaa acctctccga taataaatcc 1500
tcagaaaagc tggtcgataa aatcaaatcg tattccgttg atcagcgggg gcaggtggcg 1560
atcctgacgg atactcccgg ccgccataag atgagtatta tgccctcgct ggatgcttcc 1620
ccggagagcc atatttccct cagcctgcat tttgccgatg cccaccaggg gttattgcac 1680
gggaagtcgg agcttgaggc acaatctgtc gcgatcagcc atgggcgact ggttgtggcc 1740
gatagcgaag gcaagctgtt tagcgccgcc attccgaagc aaggggatgg aaacgaactg 1800
aaaatgaaag ccatgcctca gcatgcgctc gatgaacatt ttggtcatga ccaccagatt 1860
tctggatttt tccatgacga ccacggccag cttaatgcgc tggtgaaaaa taacttcagg 1920
cagcagcatg cctgcccgtt gggtaacgat catcagtttc accccggctg gaacctgact 1980
gatgcgctgg ttatcgacaa tcagctgggg ctgcatcata ccaatcctga accgcatgag 2040
attcttgata tggggcattt aggcagcctg gcgttacagg agggcaagct tcactatttt 2100
gaccagctga ccaaagggtg gactggcgcg gagtcagatt gtaagcagct gaaaaaaggc 2160
ctggatggag cagcttatct actgaaagac ggtgaagtga aacgcctgaa tattaatcag 2220
agcacctcct ctatcaagca cggaacggaa aacgtttttt cgctgccgca tgtgcgcaat 2280
aaaccggagc cgggagatgc cctgcaaggg ctgaataaag acgataaggc ccaggccatg 2340
gcggtgattg gggtaaataa atacctggcg ctgacggaaa aaggggacat tcgctccttc 2400
cagataaaac ccggcaccca gcagttggag cggccggcac aaactctcag ccgcgaaggt 2460
atcagcggcg aactgaaaga cattcatgtc gaccacaagc agaacctgta tgccttgacc 2520
cacgagggag aggtgtttca tcagccgcgt gaagcctggc agaatggtgc cgaaagcagc 2580
agctggcaca aactggcgtt gccacagagt gaaagtaagc taaaaagtct ggacatgagc 2640
catgagcaca aaccgattgc caccttgaa gacggtagcc agcatcagct gaaggctggc 2700
ggctggcacg cctatgcggc acctgaacgc gggccgctgg cggtgggtac cagcggttca 2760
caaaccgtct ttaaccgact aatgcagggg gtgaaaggca aggtgatccc aggcagcggg 2820
ttgacggtta agctctcggc tcagacgggg ggaatgaccg gcgccgaagg gcgcaaggtc 2880
agcagtaaat tttccgaaag gatccgcgcc tatgcgttca acccaacaat gtccacgccg 2940
cgaccgatta aaaatgctgc ttatgccaca cagcacggct ggcaggggcg tgaggggttg 3000
aagccgttgt acgagatgca gggagcgctg attaaacaac tggatgcgca taacgttcgt 3060
cataacgcgc cacagccaga tttgcagagc aaactggaaa ctctggattt aggcgaacat 3120
ggcgcagaat tgcttaacga catgaagcgc ttccgcgacg aactggagca gagtgcaacc 3180

cgttcggtga ccgttttagg tcaacatcag ggagtgctaa aaagcaacgg tgaaatcaat 3240
agcgaattta agccatcgcc cggcaaggcg ttggtccaga gctttaacgt caatcgctct 3300
ggtcaggatc taagcaagtc actgcaacag gcagtacatg ccacgccgcc atccgcagag 3360
agtaaactgc aatccatgct ggggcacttt gtcagtgccg gggtggatat gagtcatcag 3420
aagggcgaga tcccgctggg ccgccagcgc gatccgaatg ataaaaccgc actgaccaaa 3480
tcgcgtttaa ttttagatac cgtgaccatc ggtgaactgc atgaactggc cgataaggcg 3540
aaactggtat ctgaccataa acccgatgcc gatcagataa aacagctgcg ccagcagttc 3600
gatacgctgc gtgaaaagcg gtatgagagc aatccggtga agcattacac cgatatgggc 3660
ttcacccata ataaggcgct ggaagcaaac tatgatgcgg tcaaagcctt tatcaatgcc 3720
tttaagaaag agcaccacgg cgtcaatctg accacgcgta ccgtactgga atcacagggc 3780
agtgcggagc tggcgaagaa gctcaagaat acgctgttgt ccctggacag tggtgaaagt 3840
atgagcttca gccggtcata tggcgggggc gtcagcactg tctttgtgcc taccсttagc 3900
aagaaggtgc cagttccggt gatccccgga gccggcatca cgctggatcg cgcctataac 3960
ctgagcttca gtcgtaccag cggcggattg aacgtcagtt ttggccgcga cggcggggtg 4020
agtggtaaca tcatggtcgc taccggccat gatgtgatgc cctatatgac cggtaagaaa 4080
accagtgcag gtaacgccag tgactggttg agcgcaaaac ataaaatcag cccggacttg 4140
cgtatcggcg ctgctgtgag tggcaccctg caaggaacgc tacaaaacag cctgaagttt 4200
aagctgacag aggatgagct gcctggcttt atccatggct tgacgcatgg cacgttgacc 4260
ccggcagaac tgttgcaaaa ggggatcgaa catcagatga agcagggcag caaactgacg 4320
tttagcgtcg atacctcggc aaatctggat ctgcgtgccg gtatcaatct gaacgaagac 4380
ggcagtaaac caaatggtgt cactgcccgt gtttctgccg ggctaagtgc atcggcaaac 4440
ctggccgccg gctcgcgtga acgcagcacc acctctggcc agtttggcag cacgacttcg 4500
gccagcaata accgcccaac cttcctcaac ggggtcggcg cgggtgctaa cctgacggct 4560
gctttagggg ttgcccattc atctacgcat gaagggaaac cggtcgggat cttcccggca 4620
tttacctcga ccaatgtttc ggcagcgctg gcgctggata accgtacctc acagagtatc 4680
agcctggaat tgaagcgcgc ggagccggtg accagcaacg atatcagcga gttgacctcc 4740
acgctgggaa aacactttaa ggatagcgcc acaacgaaga tgcttgccgc tctcaaagag 4800
ttagatgacg ctaagcccgc tgaacaactg catattttac agcagcattt cagtgcaaaa 4860
gatgtcgtcg gtgatgaacg ctacgaggcg gtgcgcaacc tgaaaaaact ggtgatacgt 4920
caacaggctg cggacagcca cagcatggaa ttaggatctg ccagtcacag cacgacctac 4980
aataatctgt cgagaataaa taatgacggc attgtcgagc tgctacacaa acatttcgat 5040
gcggcattac cagcaagcag tgccaaacgt cttggtgaaa tgatgaataa cgatccggca 5100
ctgaaagata ttattaagca gctgcaaagt acgccgttca gcagcgccag cgtgtcgatg 5160
gagctgaaag atggtctgcg tgagcagacg gaaaaagcaa tactggacgg taaggtcggt 5220
cgtgaagaag tgggagtact tttccaggat cgtaacaact tgcgtgttaa atcggtcagc 5280
gtcagtcagt ccgtcagcaa aagcgaaggc ttcaataccc cagcgctgtt actggggacg 5340
agcaacagcg ctgctatgag catggagcgc aacatcggaa ccattaattt taaatacggc 5400
caggatcaga acaccccacg gcgatttacc ctggagggtg gaatagctca ggctaatccg 5460
caggtcgcat ctgcgcttac tgatttgaag aaggaagggc tggaaatgaa gagctaa 5517

<210> 8
<211> 1838
<212> PRT
<213> Erwinia amylovora

<400> 8
Met Glu Leu Lys S r Leu Gly Thr Glu His Lys Ala Ala Val His Thr

1 5 10 15

Ala Ala His Asn Pro Val Gly His Gly Val Ala Leu Gln Gln Gly Ser
20 25 30

Ser Ser Ser Ser Pro Gln Asn Ala Ala Ala Ser Leu Ala Ala Glu Gly
35 40 45

Lys Asn Arg Gly Lys Met Pro Arg Ile His Gln Pro Ser Thr Ala Ala
50 55 60

Asp Gly Ile Ser Ala Ala His Gln Gln Lys Lys Ser Phe Ser Leu Arg
65 70 75 80

Gly Cys Leu Gly Thr Lys Lys Phe Ser Arg Ser Ala Pro Gln Gly Gln
85 90 95

Pro Gly Thr Thr His Ser Lys Gly Ala Thr Leu Arg Asp Leu Leu Ala
100 105 110

Arg Asp Asp Gly Glu Thr Gln His Glu Ala Ala Ala Pro Asp Ala Ala
115 120 125

Arg Leu Thr Arg Ser Gly Gly Val Lys Arg Arg Asn Met Asp Asp Met
130 135 140

Ala Gly Arg Pro Met Val Lys Gly Gly Ser Gly Glu Asp Lys Val Pro
145 150 155 160

Thr Gln Gln Lys Arg His Gln Leu Asn Asn Phe Gly Gln Met Arg Gln
165 170 175

Thr Met Leu Ser Lys Met Ala His Pro Ala Ser Ala Asn Ala Gly Asp
180 185 190

Arg Leu Gln His Ser Pro Pro His Ile Pro Gly Ser His His Glu Ile
195 200 205

Lys Glu Glu Pro Val Gly Ser Thr Ser Lys Ala Thr Thr Ala His Ala
210 215 220

Asp Arg Val Glu Ile Ala Gln Glu Asp Asp Asp Ser Glu Phe Gln Gln
225 230 235 240

Leu His Gln Gln Arg Leu Ala Arg Glu Arg Glu Asn Pro Pro Gln Pro
245 250 255

Pro Lys Leu Gly Val Ala Thr Pro Ile Ser Ala Arg Phe Gln Pro Lys

260 265 270

Leu Thr Ala Val Ala Glu Ser Val Leu Glu Gly Thr Asp Thr Thr Gln
275 280 285

Ser Pro Leu Lys Pro Gln Ser Met Leu Lys Gly Ser Gly Ala Gly Val
290 295 300

Thr Pro Leu Ala Val Thr Leu Asp Lys Gly Lys Leu Gln Leu Ala Pro
305 310 315 320

Asp Asn Pro Pro Ala Leu Asn Thr Leu Leu Lys Gln Thr Leu Gly Lys
325 330 335

Asp Thr Gln His Tyr Leu Ala His His Ala Ser Ser Asp Gly Ser Gln
340 345 350

His Leu Leu Leu Asp Asn Lys Gly His Leu Phe Asp Ile Lys Ser Thr
355 360 365

Ala Thr Ser Tyr Ser Val Leu His Asn Ser His Pro Gly Glu Ile Lys
370 375 380

Gly Lys Leu Ala Gln Ala Gly Thr Gly Ser Val Ser Val Asp Gly Lys
385 390 395 400

Ser Gly Lys Ile Ser Leu Gly Ser Gly Thr Gln Ser His Asn Lys Thr
405 410 415

Met Leu Ser Gln Pro Gly Glu Ala His Arg Ser Leu Leu Thr Gly Ile
420 425 430

Trp Gln His Pro Ala Gly Ala Ala Arg Pro Gln Gly Glu Ser Ile Arg
435 440 445

Leu His Asp Asp Lys Ile His Ile Leu His Pro Glu Leu Gly Val Trp
450 455 460

Gln Ser Ala Asp Lys Asp Thr His Ser Gln Leu Ser Arg Gln Ala Asp
465 470 475 480

Gly Lys Leu Tyr Ala Leu Lys Asp Asn Arg Thr Leu Gln Asn Leu Ser
485 490 495

Asp Asn Lys S r Ser Glu Lys Leu Val Asp Lys Ile Lys Ser Tyr Ser
500 505 510

Val Asp Gln Arg Gly Gln Val Ala Ile Leu Thr Asp Thr Pro Gly Arg

515 520 525

His Lys Met Ser Ile Met Pro Ser Leu Asp Ala Ser Pro Glu Ser His
530 535 540

Ile Ser Leu Ser Leu His Phe Ala Asp Ala His Gln Gly Leu Leu His
545 550 555 560

Gly Lys Ser Glu Leu Glu Ala Gln Ser Val Ala Ile Ser His Gly Arg
565 570 575

Leu Val Val Ala Asp Ser Glu Gly Lys Leu Phe Ser Ala Ala Ile Pro
580 585 590

Lys Gln Gly Asp Gly Asn Glu Leu Lys Met Lys Ala Met Pro Gln His
595 600 605

Ala Leu Asp Glu His Phe Gly His Asp His Gln Ile Ser Gly Phe Phe
610 615 620

His Asp Asp His Gly Gln Leu Asn Ala Leu Val Lys Asn Asn Phe Arg
625 630 635 640

Gln Gln His Ala Cys Pro Leu Gly Asn Asp His Gln Phe His Pro Gly
645 650 655

Trp Asn Leu Thr Asp Ala Leu Val Ile Asp Asn Gln Leu Gly Leu His
660 665 670

His Thr Asn Pro Glu Pro His Glu Ile Leu Asp Met Gly His Leu Gly
675 680 685

Ser Leu Ala Leu Gln Glu Gly Lys Leu His Tyr Phe Asp Gln Leu Thr
690 695 700

Lys Gly Trp Thr Gly Ala Glu Ser Asp Cys Lys Gln Leu Lys Lys Gly
705 710 715 720

Leu Asp Gly Ala Ala Tyr Leu Leu Lys Asp Gly Glu Val Lys Arg Leu
725 730 735

Asn Ile Asn Gln Ser Thr Ser Ser Ile Lys His Gly Thr Glu Asn Val
740 745 750

Phe S r Leu Pro His Val Arg Asn Lys Pro Glu Pro Gly Asp Ala Leu
755 760 765

Gln Gly Leu Asn Lys Asp Asp Lys Ala Gln Ala Met Ala Val Il Gly

770 775 780

Val Asn Lys Tyr Leu Ala Leu Thr Glu Lys Gly Asp Ile Arg Ser Phe
785 790 795 800

Gln Ile Lys Pro Gly Thr Gln Gln Leu Glu Arg Pro Ala Gln Thr Leu
805 810 815

Ser Arg Glu Gly Ile Ser Gly Glu Leu Lys Asp Ile His Val Asp His
820 825 830

Lys Gln Asn Leu Tyr Ala Leu Thr His Glu Gly Glu Val Phe His Gln
835 840 845

Pro Arg Glu Ala Trp Gln Asn Gly Ala Glu Ser Ser Ser Trp His Lys
850 855 860

Leu Ala Leu Pro Gln Ser Glu Ser Lys Leu Lys Ser Leu Asp Met Ser
865 870 875 880

His Glu His Lys Pro Ile Ala Thr Phe Glu Asp Gly Ser Gln His Gln
885 890 895

Leu Lys Ala Gly Gly Trp His Ala Tyr Ala Ala Pro Glu Arg Gly Pro
900 905 910

Leu Ala Val Gly Thr Ser Gly Ser Gln Thr Val Phe Asn Arg Leu Met
915 920 925

Gln Gly Val Lys Gly Lys Val Ile Pro Gly Ser Gly Leu Thr Val Lys
930 935 940

Leu Ser Ala Gln Thr Gly Gly Met Thr Gly Ala Glu Gly Arg Lys Val
945 950 955 960

Ser Ser Lys Phe Ser Glu Arg Ile Arg Ala Tyr Ala Phe Asn Pro Thr
965 970 975

Met Ser Thr Pro Arg Pro Ile Lys Asn Ala Ala Tyr Ala Thr Gln His
980 985 990

Gly Trp Gln Gly Arg Glu Gly Leu Lys Pro Leu Tyr Glu Met Gln Gly
995 1000 1005

Ala L u Ile Lys Gln Leu Asp Ala His Asn Val Arg His Asn Ala Pro
1010 1015 1020

Gln Pr Asp L u Gln Ser Lys Leu Glu Thr Leu Asp Leu Gly Glu His

1025 1030 1035 1040

Gly Ala Glu Leu Leu Asn Asp Met Lys Arg Phe Arg Asp Glu Leu Glu
1045 1050 1055

Gln Ser Ala Thr Arg Ser Val Thr Val Leu Gly Gln His Gln Gly Val
1060 1065 1070

Leu Lys Ser Asn Gly Glu Ile Asn Ser Glu Phe Lys Pro Ser Pro Gly
1075 1080 1085

Lys Ala Leu Val Gln Ser Phe Asn Val Asn Arg Ser Gly Gln Asp Leu
1090 1095 1100

Ser Lys Ser Leu Gln Gln Ala Val His Ala Thr Pro Pro Ser Ala Glu
1105 1110 1115 1120

Ser Lys Leu Gln Ser Met Leu Gly His Phe Val Ser Ala Gly Val Asp
1125 1130 1135

Met Ser His Gln Lys Gly Glu Ile Pro Leu Gly Arg Gln Arg Asp Pro
1140 1145 1150

Asn Asp Lys Thr Ala Leu Thr Lys Ser Arg Leu Ile Leu Asp Thr Val
1155 1160 1165

Thr Ile Gly Glu Leu His Glu Leu Ala Asp Lys Ala Lys Leu Val Ser
1170 1175 1180

Asp His Lys Pro Asp Ala Asp Gln Ile Lys Gln Leu Arg Gln Gln Phe
1185 1190 1195 1200

Asp Thr Leu Arg Glu Lys Arg Tyr Glu Ser Asn Pro Val Lys His Tyr
1205 1210 1215

Thr Asp Met Gly Phe Thr His Asn Lys Ala Leu Glu Ala Asn Tyr Asp
1220 1225 1230

Ala Val Lys Ala Phe Ile Asn Ala Phe Lys Lys Glu His His Gly Val
1235 1240 1245

Asn Leu Thr Thr Arg Thr Val Leu Glu Ser Gln Gly Ser Ala Glu Leu
1250 1255 1260

Ala Lys Lys Leu Lys Asn Thr Leu L u Ser Leu Asp Ser Gly Glu Ser
1265 1270 1275 1280

Met S r Phe Ser Arg Ser Tyr Gly Gly Gly Val Ser Thr Val Phe Val

1285 1290 1295

Pro Thr Leu Ser Lys Lys Val Pro Val Pro Val Ile Pro Gly Ala Gly
1300 1305 1310

Ile Thr Leu Asp Arg Ala Tyr Asn Leu Ser Phe Ser Arg Thr Ser Gly
1315 1320 1325

Gly Leu Asn Val Ser Phe Gly Arg Asp Gly Gly Val Ser Gly Asn Ile
1330 1335 1340

Met Val Ala Thr Gly His Asp Val Met Pro Tyr Met Thr Gly Lys Lys
1345 1350 1355 1360

Thr Ser Ala Gly Asn Ala Ser Asp Trp Leu Ser Ala Lys His Lys Ile
1365 1370 1375

Ser Pro Asp Leu Arg Ile Gly Ala Ala Val Ser Gly Thr Leu Gln Gly
1380 1385 1390

Thr Leu Gln Asn Ser Leu Lys Phe Lys Leu Thr Glu Asp Glu Leu Pro
1395 1400 1405

Gly Phe Ile His Gly Leu Thr His Gly Thr Leu Thr Pro Ala Glu Leu
1410 1415 1420

Leu Gln Lys Gly Ile Glu His Gln Met Lys Gln Gly Ser Lys Leu Thr
1425 1430 1435 1440

Phe Ser Val Asp Thr Ser Ala Asn Leu Asp Leu Arg Ala Gly Ile Asn
1445 1450 1455

Leu Asn Glu Asp Gly Ser Lys Pro Asn Gly Val Thr Ala Arg Val Ser
1460 1465 1470

Ala Gly Leu Ser Ala Ser Ala Asn Leu Ala Ala Gly Ser Arg Glu Arg
1475 1480 1485

Ser Thr Thr Ser Gly Gln Phe Gly Ser Thr Thr Ser Ala Ser Asn Asn
1490 1495 1500

Arg Pro Thr Phe Leu Asn Gly Val Gly Ala Gly Ala Asn Leu Thr Ala
1505 1510 1515 1520

Ala Leu Gly Val Ala His Ser Ser Thr His Glu Gly Lys Pro Val Gly
1525 1530 1535

Ile Phe Pro Ala Phe Thr Ser Thr Asn Val Ser Ala Ala Leu Ala Leu

1540 1545 1550

Asp Asn Arg Thr Ser Gln Ser Ile Ser Leu Glu Leu Lys Arg Ala Glu
1555 1560 1565

Pro Val Thr Ser Asn Asp Ile Ser Glu Leu Thr Ser Thr Leu Gly Lys
1570 1575 1580

His Phe Lys Asp Ser Ala Thr Thr Lys Met Leu Ala Ala Leu Lys Glu
1585 1590 1595 1600

Leu Asp Asp Ala Lys Pro Ala Glu Gln Leu His Ile Leu Gln Gln His
1605 1610 1615

Phe Ser Ala Lys Asp Val Val Gly Asp Glu Arg Tyr Glu Ala Val Arg
1620 1625 1630

Asn Leu Lys Lys Leu Val Ile Arg Gln Gln Ala Ala Asp Ser His Ser
1635 1640 1645

Met Glu Leu Gly Ser Ala Ser His Ser Thr Thr Tyr Asn Asn Leu Ser
1650 1655 1660

Arg Ile Asn Asn Asp Gly Ile Val Glu Leu Leu His Lys His Phe Asp
1665 1670 1675 1680

Ala Ala Leu Pro Ala Ser Ser Ala Lys Arg Leu Gly Glu Met Met Asn
1685 1690 1695

Asn Asp Pro Ala Leu Lys Asp Ile Ile Lys Gln Leu Gln Ser Thr Pro
1700 1705 1710

Phe Ser Ser Ala Ser Val Ser Met Glu Leu Lys Asp Gly Leu Arg Glu
1715 1720 1725

Gln Thr Glu Lys Ala Ile Leu Asp Gly Lys Val Gly Arg Glu Glu Val
1730 1735 1740

Gly Val Leu Phe Gln Asp Arg Asn Asn Leu Arg Val Lys Ser Val Ser
1745 1750 1755 1760

Val Ser Gln Ser Val Ser Lys Ser Glu Gly Phe Asn Thr Pro Ala Leu
1765 1770 1775

Leu L u Gly Thr Ser Asn S r Ala Ala M t Ser Met Glu Arg Asn Ile
1780 1785 1790

Gly Thr Ile Asn Phe Lys Tyr Gly Gln Asp Gln Asn Thr Pro Arg Arg

1795 1800 1805

Phe Thr Leu Glu Gly Gly Ile Ala Gln Ala Asn Pro Gln Val Ala Ser
1810 1815 1820

Ala Leu Thr Asp Leu Lys Lys Glu Gly Leu Glu Met Lys Ser
1825 1830 1835

<210> 9
<211> 420
<212> DNA
<213> Erwinia amylovora

<400> 9
atgacatcgt cacagcagcg ggttgaaagg tttttacagt atttctccgc cgggtgtaaa 60
acgcccatac atctgaaaga cggggtgtgc gccctgtata acgaacaaga tgaggaggcg 120
gcggtgctgg aagtaccgca acacagcgac agcctgttac tacactgccg aatcattgag 180
gctgacccac aaacttcaat aaccctgtat tcgatgctat tacagctgaa ttttgaaatg 240
gcggccatgc gcggctgttg gctggcgctg gatgaactgc acaacgtgcg tttatgtttt 300
cagcagtcgc tggagcatct ggatgaagca agttttagcg atatcgttag cggcttcatc 360
gaacatgcgg cagaagtgcg tgagtatata gcgcaattag acgagagtag cgcggcataa 420

<210> 10
<211> 139
<212> PRT
<213> Erwinia amylovora

<400> 10
Met Thr Ser Ser Gln Gln Arg Val Glu Arg Phe Leu Gln Tyr Phe Ser
1 5 10 15

Ala Gly Cys Lys Thr Pro Ile His Leu Lys Asp Gly Val Cys Ala Leu
20 25 30

Tyr Asn Glu Gln Asp Glu Glu Ala Ala Val Leu Glu Val Pro Gln His
35 40 45

Ser Asp Ser Leu Leu Leu His Cys Arg Ile Ile Glu Ala Asp Pro Gln
50 55 60

Thr Ser Ile Thr Leu Tyr Ser Met Leu Leu Gln Leu Asn Phe Glu Met
65 70 75 80

Ala Ala Met Arg Gly Cys Trp Leu Ala Leu Asp Glu Leu His Asn Val
85 90 95

Arg Leu Cys Phe Gln Gln Ser Leu Glu His Leu Asp Glu Ala Ser Phe
100 105 110

Ser Asp Ile Val Ser Gly Phe Ile Glu His Ala Ala Glu Val Arg Glu
115 120 125

Tyr Ile Ala Gln Leu Asp Glu Ser Ser Ala Ala
130 135

<210> 11
<211> 341
<212> PRT
<213> Pseudomonas syringae

<400> 11
Met Gln Ser Leu Ser Leu Asn Ser Ser Ser Leu Gln Thr Pro Ala Met
1 5 10 15

Ala Leu Val Leu Val Arg Pro Glu Ala Glu Thr Thr Gly Ser Thr Ser
20 25 30

Ser Lys Ala Leu Gln Glu Val Val Val Lys Leu Ala Glu Glu Leu Met
35 40 45

Arg Asn Gly Gln Leu Asp Asp Ser Ser Pro Leu Gly Lys Leu Leu Ala
50 55 60

Lys Ser Met Ala Ala Asp Gly Lys Ala Gly Gly Gly Ile Glu Asp Val
65 70 75 80

Ile Ala Ala Leu Asp Lys Leu Ile His Glu Lys Leu Gly Asp Asn Phe
85 90 95

Gly Ala Ser Ala Asp Ser Ala Ser Gly Thr Gly Gln Gln Asp Leu Met
100 105 110

Thr Gln Val Leu Asn Gly Leu Ala Lys Ser Met Leu Asp Asp Leu Leu
115 120 125

Thr Lys Gln Asp Gly Gly Thr Ser Phe Ser Glu Asp Asp Met Pro Met
130 135 140

Leu Asn Lys Ile Ala Gln Phe Met Asp Asp Asn Pro Ala Gln Phe Pro
145 150 155 160

Lys Pro Asp Ser Gly Ser Trp Val Asn Glu Leu Lys Glu Asp Asn Phe
165 170 175

Leu Asp Gly Asp Glu Thr Ala Ala Phe Arg Ser Ala Leu Asp Ile Ile
180 185 190

Gly Gln Gln Leu Gly Asn Gln Gln Ser Asp Ala Gly Ser Leu Ala Gly
195 200 205

Thr Gly Gly Gly Leu Gly Thr Pro Ser Ser Phe Ser Asn Asn Ser Ser
210 215 220

Val Met Gly Asp Pro Leu Ile Asp Ala Asn Thr Gly Pro Gly Asp Ser
225 230 235 240

Gly Asn Thr Arg Gly Glu Ala Gly Gln Leu Ile Gly Glu Leu Ile Asp
245 250 255

Arg Gly Leu Gln Ser Val Leu Ala Gly Gly Gly Leu Gly Thr Pro Val
260 265 270

Asn Thr Pro Gln Thr Gly Thr Ser Ala Asn Gly Gly Gln Ser Ala Gln
275 280 285

Asp Leu Asp Gln Leu Leu Gly Gly Leu Leu Leu Lys Gly Leu Glu Ala
290 295 300

Thr Leu Lys Asp Ala Gly Gln Thr Gly Thr Asp Val Gln Ser Ser Ala
305 310 315 320

Ala Gln Ile Ala Thr Leu Leu Val Ser Thr Leu Leu Gln Gly Thr Arg
325 330 335

Asn Gln Ala Ala Ala
340

<210> 12
<211> 1026
<212> DNA
<213> Pseudomonas syringae

<400> 12
atgcagagtc tcagtcttaa cagcagctcg ctgcaaaccc cggcaatggc ccttgtcctg 60
gtacgtcctg aagccgagac gactggcagt acgtcgagca aggcgcttca ggaagttgtc 120
gtgaagctgg ccgaggaact gatgcgcaat ggtcaactcg acgacagctc gccattggga 180
aaactgttgg ccaagtcgat ggccgcagat ggcaaggcgg gcggcggtat tgaggatgtc 240
atcgctgcgc tggacaagct gatccatgaa aagctcggtg acaacttcgg cgcgtctgcg 300
gacagcgcct cgggtaccgg acagcaggac ctgatgactc aggtgctcaa tggcctggcc 360
aagtcgatgc tcgatgatct tctgaccaag caggatggcg ggacaagctt ctccgaagac 420

gatatgccga tgctgaacaa gatcgcgcag ttcatggatg acaatcccgc acagtttccc 480
aagccggact cgggctcctg ggtgaacgaa ctcaaggaag acaacttcct tgatggcgac 540
gaaacggctg cgttccgttc ggcactcgac atcattggcc agcaactggg taatcagcag 600
agtgacgctg gcagtctggc agggacgggt ggaggtctgg gcactccgag cagtttttcc 660
aacaactcgt ccgtgatggg tgatccgctg atcgacgcca ataccggtcc cggtgacagc 720
ggcaataccc gtggtgaagc ggggcaactg atcggcgagc ttatcgaccg tggcctgcaa 780
tcggtattgg ccggtggtgg actgggcaca cccgtaaaca ccccgcagac cggtacgtcg 840
gcgaatggcg gacagtccgc tcaggatctt gatcagttgc tgggcggctt gctgctcaag 900
ggcctggagg caacgctcaa ggatgccggg caaacaggca ccgacgtgca gtcgagcgct 960
gcgcaaatcg ccaccttgct ggtcagtacg ctgctgcaag gcacccgcaa tcaggctgca 1020
gcctga 1026

<210> 13
<211> 1729
<212> DNA
<213> Pseudomonas syringae

<400> 13
tccacttcgc tgattttgaa attggcagat tcatagaaac gttcaggtgt ggaaatcagg 60
ctgagtgcgc agatttcgtt gataagggtg tggtactggt cattgttggt catttcaagg 120
cctctgagtg cggtgcggag caataccagt cttcctgctg gcgtgtgcac actgagtcgc 180
aggcataggc atttcagttc cttgcgttgg ttgggcatat aaaaaaagga acttttaaaa 240
acagtgcaat gagatgccgg caaaacggga accggtcgct gcgctttgcc actcacttcg 300
agcaagctca accccaaaca tccacatccc tatcgaacgg acagcgatac ggccacttgc 360
tctggtaaac cctggagctg gcgtcggtcc aattgcccac ttagcgaggt aacgcagcat 420
gagcatcggc atcacacccc ggccgcaaca gaccaccacg ccactcgatt tttcggcgct 480
aagcggcaag agtcctcaac caaacacgtt cggcgagcag aacactcagc aagcgatcga 540
cccgagtgca ctgttgttcg gcagcgacac acagaaagac gtcaacttcg gcacgcccga 600
cagcaccgtc cagaatccgc aggacgccag caagcccaac gacagccagt ccaacatcgc 660
taaattgatc agtgcattga tcatgtcgtt gctgcagatg ctcaccaact ccaataaaaa 720
gcaggacacc aatcaggaac agcctgatag ccaggctcct ttccagaaca acggcgggct 780
cggtacaccg tcggccgata gcgggggcgg cggtacaccg gatgcgacag gtggcggcgg 840
cggtgatacg ccaagcgcaa caggcggtgg cggcggtgat actccgaccg caacaggcgg 900
tggcggcagc ggtggcggcg gcacacccac tgcaacaggt ggcggcagcg gtggcacacc 960
cactgcaaca ggcggtggcg agggtggcgt aacaccgcaa atcactccgc agttggccaa 1020
ccctaaccgt acctcaggta ctggctcggt gtcggacacc gcaggttcta ccgagcaagc 1080
cggcaagatc aatgtggtga aagacaccat caaggtcggc gctggcgaag tctttgacgg 1140
ccacggcgca accttcactg ccgacaaatc tatgggtaac ggagaccagg gcgaaaatca 1200
gaagcccatg ttcgagctgg ctgaaggcgc tacgttgaag aatgtgaacc tgggtgagaa 1260
cgaggtcgat ggcatccacg tgaaagccaa aaacgctcag gaagtcacca ttgacaacgt 1320
gcatgcccag aacgtcggtg aagacctgat tacggtcaaa ggcgagggag gcgcagcggt 1380
cactaatctg aacatcaaga acagcagtgc caaaggtgca gacgacaagg ttgtccagct 1440
caacgccaac actcacttga aaatcgacaa cttcaaggcc gacgatttcg gcacgatggt 1500
tcgcaccaac ggtggcaagc agtttgatga catgagcatc gagctgaacg gcatcgaagc 1560
taaccacggc aagttcgccc tggtgaaaag cgacagtgac gatctgaagc tggcaacggg 1620
caacatcgcc atgaccgacg tcaaacacgc ctacgataaa acccaggcat cgacccaaca 1680
caccgagctt tgaatccaga caagtagctt gaaaaaaggg ggtggactc 1729

<210> 14
<211> 424
<212> PRT
<213> Pseudomonas syringae

<400> 14
Met Ser Ile Gly Ile Thr Pro Arg Pro Gln Gln Thr Thr Thr Pro Leu
1 5 10 15

Asp Phe Ser Ala Leu Ser Gly Lys Ser Pro Gln Pro Asn Thr Phe Gly
20 25 30

Glu Gln Asn Thr Gln Gln Ala Ile Asp Pro Ser Ala Leu Leu Phe Gly
35 40 45

Ser Asp Thr Gln Lys Asp Val Asn Phe Gly Thr Pro Asp Ser Thr Val
50 55 60

Gln Asn Pro Gln Asp Ala Ser Lys Pro Asn Asp Ser Gln Ser Asn Ile
65 70 75 80

Ala Lys Leu Ile Ser Ala Leu Ile Met Ser Leu Leu Gln Met Leu Thr
85 90 95

Asn Ser Asn Lys Lys Gln Asp Thr Asn Gln Glu Gln Pro Asp Ser Gln
100 105 110

Ala Pro Phe Gln Asn Asn Gly Gly Leu Gly Thr Pro Ser Ala Asp Ser
115 120 125

Gly Gly Gly Gly Thr Pro Asp Ala Thr Gly Gly Gly Gly Gly Asp Thr
130 135 140

Pro Ser Ala Thr Gly Gly Gly Gly Gly Asp Thr Pro Thr Ala Thr Gly
145 150 155 160

Gly Gly Gly Ser Gly Gly Gly Gly Thr Pro Thr Ala Thr Gly Gly Gly
165 170 175

Ser Gly Gly Thr Pro Thr Ala Thr Gly Gly Gly Glu Gly Gly Val Thr
180 185 190

Pro Gln Ile Thr Pr Gln L u Ala Asn Pro Asn Arg Thr Ser Gly Thr
195 200 205

Gly Ser Val Ser Asp Thr Ala Gly S r Thr Glu Gln Ala Gly Lys Ile

210 215 220

Asn Val Val Lys Asp Thr Ile Lys Val Gly Ala Gly Glu Val Phe Asp
225 230 235 240

Gly His Gly Ala Thr Phe Thr Ala Asp Lys Ser Met Gly Asn Gly Asp
245 250 255

Gln Gly Glu Asn Gln Lys Pro Met Phe Glu Leu Ala Glu Gly Ala Thr
260 265 270

Leu Lys Asn Val Asn Leu Gly Glu Asn Glu Val Asp Gly Ile His Val
275 280 285

Lys Ala Lys Asn Ala Gln Glu Val Thr Ile Asp Asn Val His Ala Gln
290 295 300

Asn Val Gly Glu Asp Leu Ile Thr Val Lys Gly Glu Gly Gly Ala Ala
305 310 315 320

Val Thr Asn Leu Asn Ile Lys Asn Ser Ser Ala Lys Gly Ala Asp Asp
325 330 335

Lys Val Val Gln Leu Asn Ala Asn Thr His Leu Lys Ile Asp Asn Phe
340 345 350

Lys Ala Asp Asp Phe Gly Thr Met Val Arg Thr Asn Gly Gly Lys Gln
355 360 365

Phe Asp Asp Met Ser Ile Glu Leu Asn Gly Ile Glu Ala Asn His Gly
370 375 380

Lys Phe Ala Leu Val Lys Ser Asp Ser Asp Asp Leu Lys Leu Ala Thr
385 390 395 400

Gly Asn Ile Ala Met Thr Asp Val Lys His Ala Tyr Asp Lys Thr Gln
405 410 415

Ala Ser Thr Gln His Thr Glu Leu
420

<210> 15
<211> 344
<212> PRT
<213> Pseud monas s lanacearum

<400> 15

Met Ser Val Gly Asn Ile Gln Ser Pro Ser Asn Leu Pro Gly Leu Gln
1 5 10 15

Asn Leu Asn Leu Asn Thr Asn Thr Asn Ser Gln Gln Ser Gly Gln Ser
20 25 30

Val Gln Asp Leu Ile Lys Gln Val Glu Lys Asp Ile Leu Asn Ile Ile
35 40 45

Ala Ala Leu Val Gln Lys Ala Ala Gln Ser Ala Gly Gly Asn Thr Gly
50 55 60

Asn Thr Gly Asn Ala Pro Ala Lys Asp Gly Asn Ala Asn Ala Gly Ala
65 70 75 80

Asn Asp Pro Ser Lys Asn Asp Pro Ser Lys Ser Gln Ala Pro Gln Ser
85 90 95

Ala Asn Lys Thr Gly Asn Val Asp Asp Ala Asn Asn Gln Asp Pro Met
100 105 110

Gln Ala Leu Met Gln Leu Leu Glu Asp Leu Val Lys Leu Leu Lys Ala
115 120 125

Ala Leu His Met Gln Gln Pro Gly Gly Asn Asp Lys Gly Asn Gly Val
130 135 140

Gly Gly Ala Asn Gly Ala Lys Gly Ala Gly Gly Gln Gly Gly Leu Ala
145 150 155 160

Glu Ala Leu Gln Glu Ile Glu Gln Ile Leu Ala Gln Leu Gly Gly Gly
165 170 175

Gly Ala Gly Ala Gly Gly Ala Gly Gly Gly Val Gly Gly Ala Gly Gly
180 185 190

Ala Asp Gly Gly Ser Gly Ala Gly Gly Ala Gly Gly Ala Asn Gly Ala
195 200 205

Asp Gly Gly Asn Gly Val Asn Gly Asn Gln Ala Asn Gly Pro Gln Asn
210 215 220

Ala Gly Asp Val Asn Gly Ala Asn Gly Ala Asp Asp Gly Ser Glu Asp
225 230 235 240

Gln Gly Gly Leu Thr Gly Val Leu Gln Lys Leu Met Lys Ile Leu Asn
245 250 255

Ala Leu Val Gln Met Met Gln Gln Gly Gly Leu Gly Gly Gly Asn Gln
260 265 270

Ala Gln Gly Gly Ser Lys Gly Ala Gly Asn Ala Ser Pro Ala Ser Gly
275 280 285

Ala Asn Pro Gly Ala Asn Gln Pro Gly Ser Ala Asp Asp Gln Ser Ser
290 295 300

Gly Gln Asn Asn Leu Gln Ser Gln Ile Met Asp Val Val Lys Glu Val
305 310 315 320

Val Gln Ile Leu Gln Gln Met Leu Ala Ala Gln Asn Gly Gly Ser Gln
325 330 335

Gln Ser Thr Ser Thr Gln Pro Met
340

<210> 16
<211> 1035
<212> DNA
<213> Pseudomonas solanacearum

<400> 16
atgtcagtcg gaaacatcca gagcccgtcg aacctcccgg gtctgcagaa cctgaacctc 60
aacaccaaca ccaacagcca gcaatcgggc cagtccgtgc aagacctgat caagcaggtc 120
gagaaggaca tcctcaacat catcgcagcc ctcgtgcaga aggccgcaca gtcggcgggc 180
ggcaacaccg gtaacaccgg caacgcgccg gcgaaggacg gcaatgccaa cgcgggcgcc 240
aacgacccga gcaagaacga cccgagcaag agccaggctc cgcagtcggc caacaagacc 300
ggcaacgtcg acgacgccaa caaccaggat ccgatgcaag cgctgatgca gctgctggaa 360
gacctggtga agctgctgaa ggcggccctg cacatgcagc agcccggcgg caatgacaag 420
ggcaacggcg tgggcggtgc caacggcgcc aagggtgccg gcggccaggg cggcctggcc 480
gaagcgctgc aggagatcga gcagatcctc gcccagctcg gcggcggcgg tgctggcgcc 540
ggcggcgcgg gtggcggtgt cggcggtgct ggtggcgcgg atggcggctc cggtgcgggt 600
ggcgcaggcg gtgcgaacgg cgccgacggc ggcaatggcg tgaacggcaa ccaggcgaac 660
ggcccgcaga acgcaggcga tgtcaacggt gccaacggcg cggatgacgg cagcgaagac 720
cagggcggcc tcaccggcgt gctgcaaaag ctgatgaaga tcctgaacgc gctggtgcag 780
atgatgcagc aaggcggcct cggcggcggc aaccaggcgc agggcggctc gaagggtgcc 840
ggcaacgcct cgccggcttc cggcgcgaac ccgggcgcga accagcccgg ttcggcggat 900
gatcaatcgt ccggccagaa caatctgcaa tcccagatca tggatgtggt gaaggaggtc 960
gtccagatcc tgcagcagat gctggcggcg cagaacggcg gcagccagca gtccacctcg 1020
acgcagccga tgtaa 1035

<210> 17
<211> 10
<212> PRT

<213> Xanthomonas campestris

<400> 17
Met Asp Gly Ile Gly Asn His Phe Ser Asn
1 5 10

<210> 18
<211> 20
<212> PRT
<213> Xanthomonas campestris pv. pelargonii

<400> 18
Ser Ser Gln Gln Ser Pro Ser Ala Gly Ser Glu Gln Gln Leu Asp Gln
1 5 10 15

Leu Leu Ala Met
20

HARPIN FROM *ERWINIA AMYLOVORA* INDUCES PLANT RESISTANCE

Z.-M. Wei and S. V. Beer
Department of Plant Pathology
Cornell University
Ithaca, NY 14853
USA

Plants have evolved a complex array of biochemical pathways that enable them to recognize and respond to signals from the environment. A common form of plant resistance is the restriction of pathogen proliferation to a small zone surrounding the site of infection. Typically this restriction is accompanied by localized necrosis. In addition to local defense response, plants also respond to infection by activating defenses in uninfected parts of the plant, which result in resistance of the plant to secondary infection (Dean and Kuc, 1985). Collectively, this phenomenon of induced resistance is called systemic acquired resistance (SAR). SAR reduces the severity of disease caused by all classes of pathogens and it can persist for several weeks or longer. SAR can be induced by abiotic agents, such as salicylic acid as well as biotic agents, such as virulent and avirulent pathogens (Dean and Kuc, 1985; Malamy *et al.*, 1990). Salicylic acid is believed to play a signal function in the induction of SAR since endogenous levels of salicylic acid increase after "immunization" with an incompatible pathogen. However at present, little is known about the signal transduction pathways activated during responses of a plant to attack by a pathogen, although this knowledge is central to understanding disease susceptibility and resistance.

Erwinia amylovora is an often devastating plant pathogenic bacterium that causes the fire blight disease of pear, apple and many other rosaceous plants. In non-host plants, *E. amylovora* elicits the hypersensitive response (HR), which is characterized by a rapid, localized death of tissues infiltrated with high concentrations of bacterial cells ($>10^7$ cfu/ml) (Klement, 1982). *hrp* genes are essential for *E. amylovora* to cause disease in host plants and to elicit the HR in non-host plants (Beer *et al.*, 1991). Harpin is a heat-stable, glycine-rich, secreted protein with molecular mass of 37 kD. It is encoded by *hrpN* of *E. amylovora* (Wei *et al.*, 1992). When infiltrated into intercellular spaces, harpin elicits the HR in many plants including tobacco, pepper, sunflower, tomato cabbage, arabidopsis, cucumber, geranium, watermelon and lettuce.

The HR is believed to be associated with plant defense against pathogens. Hence, we reasoned that harpin-induced HR may induce plant resistance. We tested harpin-induced resistance in more than seven different plants against eight diseases caused by fungi, bacteria and viruses. All tested plants showed some resistance. Here we report evidence of harpin-induced resistance to three diseases, southern bacterial wilt of tomato, tobacco mosaic virus and Gliocladium leaf spot of cucumber.

Harpin-induced resistance in tomato against southern bacterial wilt caused by *Pseudomonas solanacearum*.

100 μl of a cell suspension of ca. 10^8 cfu/ml of *Escherichia coli* DH5α(pCPP430) or 100 µl of a 200 µg/ml crude harpin preparations were infiltrated into portions of the two lower true leaves of two-week-old tomato seedlings grown in 8 x 15 cm flats in the greenhouse. Twenty plants were used for each treatment. Necrosis was evident 24 hours after infiltration of harpin or *E. coli* DH5α(pCPP430), which produces and secretes

harpin. Four days after the tomato seedlings had been treated with harpin or bacteria, they were inoculated with *P. solanacearum* K60 (10^7 cfu/ml) by root dipping for three minutes. The inoculated plants were replanted into the same flats and left in a greenhouse. None of the 20 harpin-infiltrated plants showed any symptoms one week after inoculation with *P. solanacearum* K60. However, seven of the 20 buffer-infiltrated plants were stunted. After two weeks, 11 buffer-infiltrated plants showed severe wilting and five were stunted, characteristics of the southern bacterial wilt disease. In comparison, only two harpin-treated plants appeared wilted and three plants were stunted. Similar induced resistance was observed following infiltration of living bacteria *E. coli* DH5α(pCPP430), but not by *E. coli* DH5α(pCPP430⁻), which is a harpin-deficient mutant created by transposon Tn*5tac* insertion into the *hrpN* gene. These results indicate that harpin, which is produced and secreted by *hrp* gene cluster of *E. amylovora*, is responsible for the induced-resistance realized.

Harpin-induced resistance in tobacco to tobacco mosaic virus (TMV)

One panel of a lower leaf of four-week-old tobacco seedlings (*Nicotiana tabacum* L. "Xanthi" with *N* gene) was infiltrated with 100 µl of a 200 µg/ml crude harpin preparation in 5 mM phosphate buffer. Three days later, the plants were challenged with TMV. Fifty µl of a suspension of TMV (5 µg/ml) was rubbed on one upper leaf with 400-mesh carborundum. Six plants were used for each treatment. Necrotic lesions appeared on inoculated leaves of both harpin- and buffer-treated plants 4 days after inoculation. The average number of necrotic lesions from the six harpin-treated plants was 21, which was significantly less than the 67 lesion average that developed on six buffer-treated plants. More importantly, the size of the lesions on buffer-treated plants was larger than those on the harpin-treated plants. Actually, it was difficult to distinguish individual lesions on the buffer-treated plants by day 10, because several necrotic lesions had merged.

Harpin-induced resistance against Gliocladium leaf spot of cucumber

Harpin or a cell suspension of *E. coli* DH5α(pCPP430) was infiltrated into first two true leaves of two-week-old cucumber seedlings. Six plants were infiltrated for each treatment. Four days after infiltration of harpin, a *Gliocladium cucurbitae* spore suspension (10^6 spores/ml) was sprayed onto the whole plants. The inoculated plants were incubated in a moisture chamber. Ten days after the inoculation, typical leaf spots appeared. A mean of six lesions was present on the lowest leaves of six harpin-treated plants, but 32 lesions formed on the same leaves of the six buffer-treated plants. On the third lowest leaves, the difference in disease severity was even greater; there were virtually no lesions on harpin-treated plants, however, more than 30 lesions were found on the buffer-treated plants. Later, most of the diseased leaves on buffer-treated plants wilted and died.

The examples outlined above show that harpin is able to induce resistance in different plants against bacterial, viral and fungal pathogens. Although mechanisms of harpin-induced resistance are unknown, some of our preliminary experiments have shown that harpin may act as an elicitor of salicylic acid induction, which is believed to be involved in SAR (Malamy *et al.*, 1990). Unlike some host-specific elicitors (Keen *et al.*, 1990), harpin is able to elicit the HR on a broad range of plants. Thus, we expect that harpin-induced resistance can be achieved in many plants either by manipulation of harpin exogenously or by harpin-mediated transgenic plants.

Our studies of harpin-induced resistance are just beginning and we need to learn more to understand the exciting features of this phenomenom. For example, what is the minimal amount of harpin needed to induce plant resistance and how long does the resistance persist, and what mechanisms are involved in harpin-induced resistance? We expect that harpin as a novel molecule will play an important role in dissecting the signal transduction pathways of induced-resistance in plants, and perhaps also in practical disease control.

ACKNOWLEDGEMENT

The research reported here was sponsored in part by Eden Bioscience Inc. and the Cornell Center for Advanced Technology in Biotechnology which is sponsored by the New York State Science and Technology Foundation and industrial partners.

REFERENCES

Beer, S. V., Bauer, D. W., Jiang, X. H., Laby, R. J., Sneath, B. J., Wei, Z.-M., Wilcox, D. A. and Zumoff, C. H. 1991. The *hrp* gene cluster of *Erwinia amylovora*, p. 53-60. *in* H. Hennecke and D. P. S. Verma (eds.), Advances in Molecular Genetics of Plant-Microbe Interactions. Kluwer Academic publishers, Dordrecht.

Keen, N. T., Tamaki, S., Kobayashi, D., Gerhold, D., Stayton, M., Shen, H., Gold, S., Lorang, J., Thordal-Christensen, H., Dahlbeck, D. and Staskawicz, B. 1990. Bacteria expressing avirulence gene D produce a specific elicitor of the soybean hypersensitive reaction. Mol. Plant-Microbe Interact. 3:112-121.

Klement, Z. 1982. Hypersensitivity, p. 150-170. in M. S. Mount and G. S. Lacy (eds), Phytopathogenic Prokaryotes. Academic Press, New York.

Dean, R. A., and Kuc, J. 1985. Induced systemic protection in plants. Trends in Biotechnology. Amsterdam: Elsevier Science Publishers, B. V. May 1985. 3:125-129.

Malamy, J., Carr, J. P., Klessig, D. F. and Raskin, I. 1990. Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. Science 250:1002-1004.

van der Zwet, T., and Beer, S. V. 1995. Fire blight - its nature, prevention and control. Agriculture Information Bulletin, 681. U. S. Department of Agriculture 91 pp.

Wei, Z.-M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A. and Beer, S. V. 1992. Harpin, elicitor of hypersensitive response produced by plant pathogenic bacterium *Erwinia amylovora*. Science 257:85-88.

The Plant Journal (1996) 9(4), 431–439

Induction of systemic acquired resistance in cucumber by *Pseudomonas syringae* pv. *syringae* 61 $HrpZ_{Pss}$ protein

N.E. Strobel[1], C. Ji[1], S. Gopalan[2], J.A. Kuc[1] and S.Y. He[1,2,*]
[1]*Department of Plant Pathology, University of Kentucky, Lexington, KY 40546, USA, and*
[2]*MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824-1312, USA*

Summary

Systemic acquired resistance (SAR) is an inducible plant defense response and is effective against a broad spectrum of pathogens. Biological induction of SAR usually follows plant cell death resulting from the plant hypersensitive response (HR) elicited by an avirulent pathogen or from disease necrosis caused by a virulent pathogen. The elicitation of the HR and disease necroses by pathogenic bacteria is controlled by *hrp* genes. Previously, it was shown that the *Pseudomonas syringae* 61 (Pss61) $HrpZ_{Pss}$ protein (formally $harpin_{Pss}$) elicited the HR in plants. In this study, it is shown that $HrpZ_{Pss}$ induced SAR in cucumber to diverse pathogens, including the anthracnose fungus (*Colletotrichum lagenarium*), tobacco necrosis virus and the bacterial angular leaf spot bacterium (*P. s.* pv. *lachrymans*). A *hrpH* mutant of Pss61, which is defective in the secretion of $HrpZ_{Pss}$ and, possibly, other protein elicitors, failed to elicit SAR. Pathogenesis-related (PR) proteins, including peroxidase, β-glucanase and chitinases, were induced in cucumber plants inoculated with Pss61, *C. lagenarium* or $HrpZ_{Pss}$. The induction patterns of PR proteins by $HrpZ_{Pss}$ and Pss61 were the same, but were different from that induced by *C. lagenarium*. Interestingly, the *hrpH* mutant induced two of the three identified PR proteins, despite its failure to induce SAR. These results suggest that proteinaceous elicitors, such as $HrpZ_{Pss}$, that traverse the bacterial Hrp secretion pathway are involved in the biological induction of SAR and that at least some PR proteins can be induced by bacterial factors that are not controlled by *hrp* genes.

Introduction

Localized infection of plants by necrotizing pathogens can result in systemic acquired resistance (SAR) to disease, which persists for weeks to months and is effective against diverse pathogens including fungi, bacteria, and necrotizing viruses (Kuc, 1982; Ross, 1961). Biological induction of SAR is usually associated with prior plant cell death during the hypersensitive response (HR) or disease necrosis triggered by avirulent or virulent pathogens, respectively (Cameron *et al.*, 1994; Kuc, 1982; Ross, 1961; Uknes *et al.*, 1993). Certain synthetic chemicals, such as salicylic acid (SA) and 2,6-dichloroisonicotinic acid (INA), also can be very effective in the induction of SAR when applied to plants (Metraux *et al.*, 1991; White, 1979). The induction of SAR in cucumber plants by an avirulent bacterial pathogen, *Pseudomonas syringae* pv. *syringae*, appears to be dependent on bacterial *hrp* genes that are required for many plant pathogenic bacteria to elicit the HR in non-host plants or to cause disease in host plants (Smith *et al.*, 1991). The HR is a complex plant resistance reaction which involves local plant cell death and restriction of pathogens to the site of their introduction (Klement, 1982).

Recent studies have shown that most Hrp proteins are involved in the assembly of a type III protein secretion pathway (the Hrp pathway) through which bacterial pathogenesis-related proteins traverse to the extracellular milieu to initiate various plant–bacterial interactions (Fenselau, 1992; Huang *et al.*, 1992, 1995; Van Gijsegem *et al.*, 1995). One family of such proteins that have been identified are heat-stable, glycine-rich proteins: harpin of *Erwinia amylovora* (Wei *et al.*, 1992), $HrpZ_{Pss}$ (formally $harpin_{Pss}$) of *P. s.* pv. *syringae* 61 (Pss61) (He *et al.*, 1993) and PopA of *P. solanacearum* (Arlat *et al.*, 1994). Harpins and PopA were shown to elicit the HR when infiltrated into the leaf laminae of appropriate plants (Arlat *et al.*, 1994; He *et al.*, 1993; Wei *et al.*, 1992), to induce exchange of H^+ and K^+ (the 'XR') across the plasmalemma (Wei *et al.*, 1992), and to generate active oxygen species (Baker *et al.*, 1993) when added to plant cell cultures, which are all properties of the HR elicited by live bacteria.

As part of our investigation into plant responses to *P. syringae* extracellular proteins under the control of the Hrp regulatory/secretion system, we studied the involvement of $HrpZ_{Pss}$ in the biological induction of SAR by *P. s.* pv. *syringae* 61. In this paper we describe the experimental results showing that $HrpZ_{Pss}$, as well as the bacterium (Pss61) that produces it, efficiently induced SAR in cucumber to diverse pathogens, including a fungus (*Colletotrichum lagenarium*), a bacterium (*P. s.* pv. *lachrymans*) and a local lesion-forming virus (tobacco necrosis virus). The *hrpH* mutant, which is defective in the secretion of $HrpZ_{Pss}$, failed to induce SAR. Multiple pathogenesis-related (PR) proteins were detected in cucumber plants treated with $HrpZ_{Pss}$, Pss61 and *C. lagenarium*. The efficacy

Received 19 September 1995; revised 2 January 1996; accepted 9 January 1996.
*For correspondence (fax +1 517 353 9168; e-mail hes@pilot.msu.edu).

of SAR induction, resistance spectrum and patterns f PR protein induction were very similar in plants treated with $HrpZ_{Pss}$ and Pss61. Interestingly, the PR protein patterns induced by $HrpZ_{Pss}$ and Pss61 were somewhat different from that induced by *C. lagenarium*. The *hrpH* mutant, though unable to induce SAR, efficiently induced some of the well-characterized PR proteins. These results suggest that the biological induction of SAR by *P. syringae* is dependent on the bacterial proteins (such as $HrpZ_{Pss}$) which traverse the Hrp secretion pathway and that at least some PR-proteins can be induced by bacterial factors other than Hrp-controlled extracellular proteins.

Results

Symptoms on cucumber leaves treated with SAR inducers

Treatment of leaves with spores of *C. lagenarium* (a virulent, necrogenic pathogen of cucumber) resulted in the development of symptoms typically obtained with the fungus in cucumber: infiltrated areas were asymptomatic for 3–4 days, after which time tissues began to collapse and become necrotic. Lesions continued to expand for several days and developed a tan to brown pigmentation. Symptoms induced by treatments with Pss61 (an avirulent, HR necrosis-inducing pathogen) and $HrpZ_{Pss}$ varied with environmental conditions in the greenhouse. Under high levels of natural light, Pss61 and $HrpZ_{Pss}$ triggered the HR within 24 and 48 h, respectively, after infiltration. The HR was restricted to infiltrated areas and did not expand as did the necroses caused by *C. lagenarium*. Under lower natural light levels (cloudy days), tissues infiltrated with Pss61 or $HrpZ_{Pss}$ developed a weaker HR characterized by increasing chlorosis over a 3–5 day period, then necroses developed gradually and irregularly, despite supplemental illumination with sodium lamps. Infiltration with *hrpH* (which is defective in the secretion of $HrpZ_{Pss}$, He *et al.*, 1993) caused either no symptoms or a very mild chlorosis under all conditions tested. Infiltration with buffer alone caused only a small ring of white necrosis resulting from mechanical damage caused by pressure of the pipette mouth against the leaf. Interestingly, infiltration with *E. amylovora* harpin protein, which was prepared from DH5α(pCPP50) (He *et al.*, 1994) and which induced a strong HR in tobacco leaves, did not induce HR necrosis in cucumber leaves (data not shown).

SAR to C. lagenarium

We first tested to see whether $HrpZ_{Pss}$ alone could induce SAR to a well-studied fungal pathogen of cucumber, *C. lagenarium*. As shown in Table 1, $HrpZ_{Pss}$ treatment induced SAR comparable to that induced by *C. lagenarium* (approximately 90% reducti n in total necrotic area relative to buffer-treated controls) in two upper leaves which expanded subsequent to inducti n treatment. The degrees of SAR induced by $HrpZ_{Pss}$, Pss61, Pss61-*hrpH* and *C. lagenarium* in cucumber were subsequently compared. Under conditions conducive to HR development in the greenhouse (high levels of natural light due to sunny weather) both $HrpZ_{Pss}$ and Pss61 efficiently induced SAR in Leaf 2 and Leaf 3 (Table 2 and Figure 1a and b). SAR was expressed as a reduction in both the number and diameter of necrotic lesions resulting from challenge with *C. lagenarium*. Protection of Leaf 2 was comparable to that induced by *C. lagenarium*, whereas protection in Leaf 3 was weaker than that induced by the fungus. Under the conditions of this experiment, expansion of Leaf 2 and Leaf 3 occurred after the onset of the HR and necrosis incited by *C. lagenarium* infiltration. Leaf 2 was fully expanded prior to challenge-inoculation, whereas Leaf 3 was not. The *hrpH* mutant did not induce SAR (Table 2). The quality and/or quantity of light profoundly influenced the induction of both the HR and SAR in cucumber by Pss61 and $HrpZ_{Pss}$ in the greenhouse. When a similar experiment was conducted under conditions non-conducive to HR development (low levels of natural light on cloudy days), neither Pss61 nor $HrpZ_{Pss}$ induced the HR or SAR, although *C. lagenarium* incited necrotic lesions on Leaf 1 and induced SAR under these conditions (data not shown).

SAR to TNV

We next examined whether $HrpZ_{Pss}$-induced SAR would be effective against a viral pathogen. In two initial experiments, the abilities of $HrpZ_{Pss}$ and *C. lagenarium* to induce SAR to TNV were compared. $HrpZ_{Pss}$ elicited a normal HR in these experiments and induced SAR to TNV local lesion formation comparable to that induced by *C. lagenarium* (Table 3 and Figure 1c and d). We then compared the abilities of $HrpZ_{Pss}$, Pss61, *hrpH*, and *C. lagenarium* to induce SAR to TNV. Under high light conditions, $HrpZ_{Pss}$ and Pss61 elicited a normal HR and induced SAR which restricted local lesion formation by TNV to an extent similar to that of SAR induced by *C. lagenarium*. The percentage of lesion number reduction was 68% for Pss61, 67.1% for $HrpZ_{Pss}$, and 75.5% for *C. lagenarium* (Table 3). Under low natural light conditions unfavorable for HR development (see Experimental procedures), $HrpZ_{Pss}$ and Pss61 elicited a weaker degree of SAR relative to that induced by *C. lagenarium*. The percentage of lesion number reduction was 44.9% for Pss61, 46.7% for $HrpZ_{Pss}$, and 89.6% for *C. lagenarium* (Table 3). The lesion numbers observed in these independent experiments varied greatly, mainly due to the use of different TNV inoculum preparations. TNV inoculum was prepared freshly each time from cucumber

Table 1. Induction by HrpZ$_{Pss}$ and the fungal pathogen, *C. lagenarium*, of systemic acquired resistance to *C. lagenarium* in cucumber

Treatment	Leaf 2			Leaf 3		
	Lesion number	Lesion diameter (mm)	Total necrotic area (mm^2)	Lesion number	Lesion diameter (mm)	Total necrotic area (mm^2)
Buffer	18.8 ± 0.6[a]	2.0 ± 0.1	60.9 ± 7.4	18.5 ± 0.6	2.5 ± 0.3	110.2 ± 29.0
HrpZ$_{Pss}$	6.5 ± 0.9	1.1 ± 0.0	6.9 ± 1.3	9.5 ± 1.7	1.3 ± 0.1	13.4 ± 3.7
C. lagenarium	3.3 ± 0.8	1.0 ± 0.0	2.6 ± 0.6	6.5 ± 1.3	1.2 ± 0.1	7.5 ± 1.3

[a]Mean + SE of four replicate plants per treatment.
Leaf 1 of young plants was infiltrated with buffer (5 mM $MgSO_4$), or HrpZ$_{Pss}$ (80 µg ml^{-1}) in buffer, or spores of *C. lagenarium* (5×10^4 spores ml^{-1}). After 7 days, Leaf 2 and Leaf 3 were challenged with 20 droplets per leaf containing spores of *C. lagenarium*. Disease was allowed to develop for 8 days.

Table 2. Induction of systemic acquired resistance to *C. lagenarium* in cucumber by *P. s.* pv. *syringae* 61 (Pss61), HrpZ$_{Pss}$, the *hrpH* mutant of Pss61 and *C. lagenarium*

Treatment	Leaf 2			Leaf 3		
	Lesion number	Lesion diameter (mm)	Total necrotic area (mm^2)	Lesion number	Lesion diameter (mm)	Total necrotic area (mm^2)
Buffer	15.4±1.2[a]	1.6±0.2	38.9±8.3	16.2±1.0	1.8±0.1	52.0±8.1
hrpH	13.2±1.1	1.7±0.1	32.1±2.2	15.4±1.6	1.8±0.1	50.0±11.9
Pss61	5.4±0.4	1.2±0.1	7.0±1.9	9.4±1.1	1.5±0.1	21.2±6.2
HrpZ$_{Pss}$	5.0±0.5	1.2±0.1	5.9±1.4	8.6±2.5	1.6±0.2	24.4±9.1
C. lagenarium	4.0±1.2	1.3±0.3	8.4±5.3	6.4±1.4	1.4±0.2	13.2±5.0

[a]Mean ± SE of five replicate plants per treatment.
Leaf 1 of young plants was infiltrated with buffer (5 mM $MgSO_4$), bacteria (OD_{600}=0.2), HrpZ$_{Pss}$ (160 µg ml^{-1}), or spores of *C. lagenarium* (5×10^4 ml^{-1}). After 8 days, Leaf 2 and Leaf 3 were challenged with 20 droplets per leaf containing spores of *C. lagenarium*. Disease was allowed to develop for 8 days.

leaves bearing TNV lesions. In experiment 3, the *hrpH* mutant induced a low level of SAR to TNV (Table 3).

SAR to P. syringae *pv.* lacrymans

HrpZ$_{Pss}$ and *C. lagenarium* also induced SAR to the angular leaf spot bacterium, *P. s.* pv. *lacrymans*. For these experiments, cucumber plants were challenge-inoculated at 11 days (by spraying) or 17 days (by rubbing) after treatment of Leaf 1 (Table 4). Although *C. lagenarium* was a more effective treatment, HrpZ$_{Pss}$ also induced significant levels of SAR to the bacterium, reducing necrotic lesion numbers by 32 and 75%, compared with 50 and 86% for *C. lagenarium*, in the two experiments, respectively.

Induction of PR proteins

PR proteins that accumulated in treated cucumber plants were first analyzed using native polyacrylamide gel electrophoresis (PAGE). All treatments (*C. lagenarium*, Pss61 and HrpZ$_{Pss}$) that induced SAR also induced the accumulation of three PR protein bands (tentatively named PR-A, PR-B and PR-C) (Figure 2a). *C. lagenarium* induced PR-C, but not PR-A and PR-B, in systemic leaves, while Pss61 and HrpZ$_{Pss}$ induced PR-B, but not PR-A and PR-C, in systemic leaves. Treatment with buffer or *hrpH* mutant did not induce these particular PR protein bands to levels that would allow visual identification. To see whether any PR proteins with known functions were induced in these plants, protein extracts were analyzed using native PAGE coupled with enzyme (chitinase, peroxidase and β-glucanase) activity staining. As shown in Figure 2(b), all three enzymes were induced in plants treated with HrpZ$_{Pss}$, Pss61 or *C. lagenarium* in both local (treated) and systemic leaves, although induction of chitinase isoforms by Pss61 and HrpZ$_{Pss}$ in systemic leaves was variable and low. The enzyme activities were substantially higher in local leaves than in systemic leaves. Surprisingly, although the *hrpH* mutant bacterium failed to induce SAR, it efficiently induced peroxidase and chitinase, especially in treated leaves (Figure 2b). Only β-glucanase was not found to be induced to high levels in the *hrpH*-treated plants (Figure 2b). It is interesting to note that PR protein levels induced by various treatments correlated well with degrees of SAR induced by the same treatments (*C. lagenarium* >HrpZ$_{Pss}$=Pss61>*hrpH*> or = buffer).

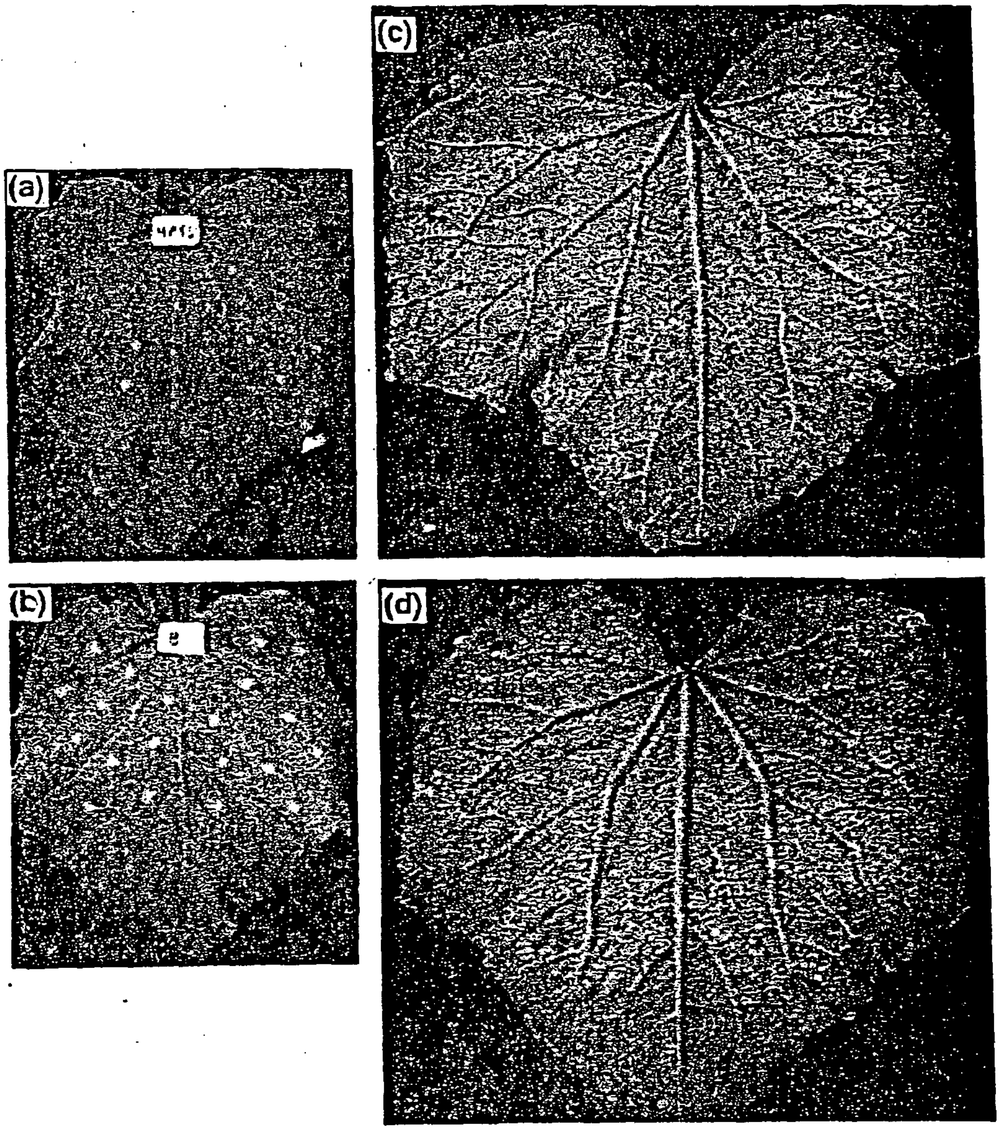


Figure 1. Disease symptoms caused by challenge-infection of *C. lagenarium* and tobacco necrosis virus on cucumber leaves with or without prior induction of SAR.
Anthracnose symptoms on Leaf 2 of cucumber plants with Leaf 1 previously treated with $HrpZ_{Pss}$ (80 µg ml^{-1}, a) or buffer (5 mM $MgSO_4$, b). Leaf 1 of young plants was infiltrated with buffer or $HrpZ_{Pss}$. After 8 days, Leaf 2 and Leaf 3 were challenged with 20 droplets per leaf containing spores of *C. lagenarium*. Disease was allowed to develop for 8 days, when the picture was taken.
TNV symptoms on Leaf 3 of cucumber plants with Leaf 1 previously treated with $HrpZ_{Pss}$ (c) or buffer (d). Leaf 1 was treated by infiltration of buffer or $HrpZ_{Pss}$ as described in footnotes to Table 1. After 7 days, Leaf 3 was challenged by mechanical inoculation with a TNV suspension prepared from infected cucumber leaves. Disease was allowed to develop for 9 days, when the picture was taken.

Induction of the pr-1 *gene and SAR in tobacco*

$HrpZ_{Pss}$ also induced SAR to tobacco mosaic virus (TMV) in tobacco (Table 5). The SAR level induced by $HrpZ_{Pss}$ was less than that induced by TMV. This was consistent with the different levels of induction of the *pr*-1 gene by $HrpZ_{Pss}$ and TMV (Figure 3). TMV-inoculated local leaves (the third and fourth true leaves) also showed more necrosis than those infiltrated with $HrpZ_{Pss}$ (data not shown), which may be partly responsible for the different levels of SAR and *pr*-1 expression in TMV- and $HrpZ_{Pss}$-induced plants.

Discussion

In this study, we show that $HrpZ_{Pss}$, a bacterial *hrp* gene product secreted via the Hrp pathway of *P. s.* pv. *syringae*, induced SAR in cucumber and tobacco. In cucumber, the

Table 3. Induction of systemic acquired resistance to TNV in cucumber by *hrpH* mutant, HrpZ$_{Pss}$, Pss61 and *C. lagenarium*

Treatment	Number of TNV necrotic local lesions			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Buffer	99.7±19.6[a]	47.2±0.9[b]	730.0±63.9[a]	342.8±34.3[a]
hrpH	-	-	556.0±53.4	324.3±11.2
HrpZ$_{Pss}$	28.7±3.8	7.5±1.2	240.4±27.5	182.8±18.8
Pss61	-	-	239.9±59.7	189.0±41.9
C. lagenarium	34.7±16.6	9.0±1.8	178.8±26.9	35.8±4.6

[a]Mean ± SE of three replicate plants per treatment. [b]Mean ± SE of eight replicate plants per treatment.
Leaf 1 was treated by infiltration of candidate inducers as described in the footnotes of Table 1. After 7 days, leaf 3 was challenged by mechanical inoculation with a TNV suspension prepared from infected cucumber leaves. Disease was allowed to develop for 10 or 9 days in experiments 1 and 2, respectively.
Experiments 1, 2 and 3 were performed under high levels of natural light during induction periods.
Experiment 4 was performed on cloudy days.

Table 4. Induction of systemic acquired resistance to *P. syringae* pv. *lacrymans* by HrpZ$_{Pss}$ and *C. lagenarium*

Treatment	Number of necrotic lesions[a]	
	Inoculated by rubbing	Inoculated by spraying
Buffer	244.8±34.2	56.6±5.9
HrpZ$_{Pss}$	168.5±24.5	13.8±1.7
C. lagenarium	122.8±9.8	8.3±2.1

[a]Mean ± SE of five replicate plants per treatment.
Leaf 1 of young plants was infiltrated with treatments as described in the footnotes of Table 1. Leaf 5 was challenged by rubbing, or by spraying the abaxial leaf surface with a suspension of bacterial cells (OD_{600}=0.2, 17 days after induction; or OD_{600}=0.1, 11 days after induction, respectively). Disease was allowed to develop for 7 or 13 days in rub-inoculated or spray-inoculated plants, respectively.

efficacy against fungal, viral and bacterial pathogens and persistence (for at least 17 days in the bacterial challenge experiments) of HrpZ$_{Pss}$-induced SAR is comparable to that induced by the bacterium (Pss61) that produces HrpZ$_{Pss}$. The degree of SAR induced in cucumber by HrpZ$_{Pss}$ was also comparable to that induced by a well-studied biological inducer of SAR, *C. lagenarium* (Kuc and Richmond, 1977). The *hrpH* mutant of *P. s.* pv. *syringae*, which is defective in the secretion of HrpZ$_{Pss}$ and other proteinaceous pathogenicity factors (He *et al.*, 1993; Huang *et al.*, 1992; Yuan *et al.*, in preparation), failed to induce SAR in cucumber. The induced PR protein patterns were the same in cucumber plants treated with Pss61 and HrpZ$_{Pss}$, but were different from that in *C. lagenarium*-treated plants. Moreover, the *hrpH* mutant, although unable to induce SAR, efficiently induced at least two well-characterized PR proteins, chitinase and peroxidase (Figure 2b). These results suggest that the biological induction of SAR and PR proteins by *P. s.* pv. *syringae* 61 in the non-host plant, cucumber, is dependent on the production and secretion of proteinaceous elicitors of the HR, such as HrpZ$_{Pss}$, but that at least some PR proteins can be induced by bacterial molecules independent of *hrp* gene functions.

The efficacy of both HrpZ$_{Pss}$ and Pss61 as inducers of SAR in cucumber appeared to be contingent upon their ability to elicit a normal HR, as low levels of natural light during the induction period, which interfered with HR development, resulted in reduced SAR to TNV and no SAR to *C. lagenarium* (Table 3; Strobel and He, unpublished work). The negative effect of low light likely resulted from an effect on HR development rather than upon the plant's capacity to express SAR because *C. lagenarium* formed necrotic lesions typical of this compatible pathogen on Leaf 1 (the inducer leaf) and triggered SAR under these same conditions. The profound effect of light on the development of the HR has been observed previously (Sequeira, 1979), although the underlying mechanism remains to be determined. The dependence of the induction of SAR on the HR is further suggested by our observations that the *hrpH* mutant of Pss61, which produces but does not secrete HR elicitors (He *et al.*, 1993), did not elicit the HR or induce SAR in cucumber. Furthermore, *E. amylovora* harpin, another HR elicitor which is structurally different from HrpZ$_{Pss}$ and which elicited a strong HR in tobacco, did not induce an HR or SAR in cucumber plants (Strobel and He, unpublished observation). In conclusion, there appears to be a tight linkage between HR development and induction of SAR in plants by avirulent bacteria.

The tight linkage between the HR and SAR suggests that the signal(s) for the induction of SAR by HrpZ$_{Pss}$ and *P. s.* pv. *syringae* 61 likely comes from dying plant cells and/or cells immediately adjacent to the dying cells during the HR. What types of cell death would lead to the induction of SAR? It has been shown that the HR triggered by live bacteria (Keen *et al.*, 1981), HrpZ$_{Pss}$ (He *et al.*, 1993) or *E. amylovora* harpin (He *et al.*, 1994) involves an active cell death pathway. Does this mean that only cells undergoing active cell death give rise to signals for SAR? The answer to this is probably not simple. SAR and PR proteins can

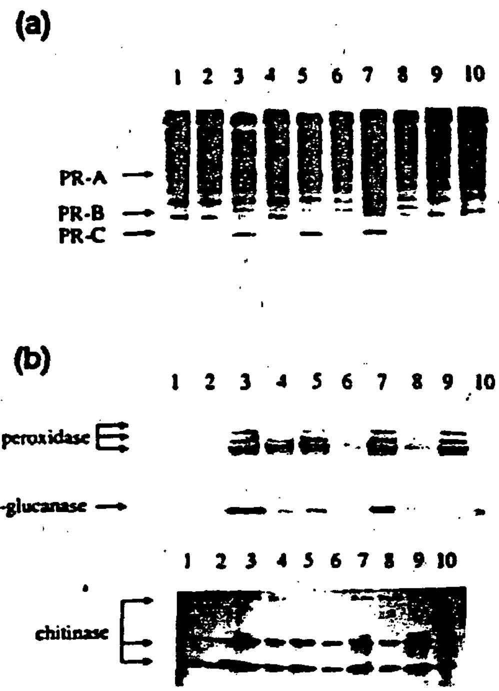


Figure 2. PR protein accumulation in cucumber plants.
PAGE (a) and PAGE coupled with activity staining (b) analyses of protein extracts from treated (lanes 1, 3, 5, 7 and 9) or systemic leaves (lanes 2, 4, 6, 8, and 10). The treatments were buffer (lanes 1 and 2), *C. lagenarium* (lanes 3 and 4), Pss61 (lanes 5 and 6), $HrpZ_{Pss}$ (lanes 7 and 8) and the *hrpH* mutant (lanes 9 and 10). PR-A, PR-B and PR-C are tentative names for the three PR proteins observed in these experiments. The identities of these PR proteins are unknown.

be induced not only by HR-eliciting avirulent pathogens, but also by necrosis-causing virulent pathogens. For example, *P. s.* pv. *lacrymans* and *C. lagenarium* can efficiently induce SAR and/or PR proteins in the susceptible host plant, cucumber (Kuc and Richmond, 1977; Smith *et al.*, 1991; this study). Unless cell death during the HR and some diseases shares the same biochemical processes, which is possible, the ability of both virulent and avirulent pathogens to induce SAR argues for multiple cell death pathways in the induction of SAR. On the other hand, not all types of plant cell death induce SAR. For example, cell death due to mechanical wounding or resulting from certain plant mutations does not induce SAR (Dietrich *et al.*, 1994). It would be important in the future to learn why certain cell death processes, but not others, lead to SAR. Endogenous signaling molecules, such as salicylic acid and H_2O_2, have been shown or suggested to be involved in the induction of SAR (Chen *et al.*, 1993; Gaffney *et al.*, 1993; Malamy *et al.*, 1990; Metraux *et al.*, 1990; Rasmussen *et al.*, 1991). However, the mechanism(s) by which various biological inducers of SAR generate these signals and the identity of the actual systemic signal(s) translocated from the induced leaves to distant leaves remain to be determined. Also, it has not been unequivocally shown that cell death is necessary for the induction of SAR.

Table 5. Induction of systemic acquired resistance to TMV by $HrpZ_{Pss}$ and TMV

	Diameter of necrotic lesions[a]
Buffer	4.41±0.05
$HrpZ_{Pss}$	3.05±0.03
TMV	2.34±0.03

[a]Mean ± SE of 100 lesions per treatment.
The third and fourth true leaves of 6-week-old tobacco plants were inoculated with TMV (100–150 lesions per leaf), or infiltrated with 120 μg ml^{-1} $harpin_{Pss}$ or 5 mM $MgSO_4$ at 10 sites (50 μl per site). Five days later the seventh and eighth true leaves were challenge-inoculated with TMV. The diameters of TMV lesions on the challenged leaves were recorded.

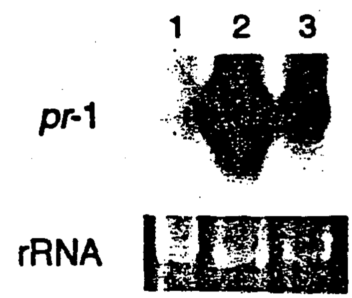


Figure 3. Induction of the *pr-1* gene in tobacco leaves.
Total RNA was isolated from systemic leaves (the ninth true leaves) of plants treated with buffer (lane 1), TMV (lane 2), or $HrpZ_{Pss}$ (lane 3) 5 days post-induction. A PCR-amplified internal fragment of the tobacco *pr-1* gene was labeled with [α-^{32}P]dATP and used as a probe. The largest rRNA species visualized after staining with ethidium bromide was used as a reference.

It is interesting to observe that, although *C. lagenarium* (a necrotizing pathogen of cucumber), Pss61 (an HR-eliciting bacterium on cucumber) and $HrpZ_{Pss}$ (an HR-eliciting protein) all induced SAR in cucumber plants, there were some differences in the induction of PR proteins by these pathogens/protein. While *C. lagenarium*, Pss61 and $HrpZ_{Pss}$ all induced PR-A, PR-B and PR-C in the inoculated leaves, only *C. lagenarium* induced PR-C in systemic leaves to a high level (visible on a PAGE gel). In contrast, PR-B was induced in systemic leaves to high levels only by $HrpZ_{Pss}$ and Pss61. The induction patterns of PR-A, PR-B, PR-C, chitinase, peroxidase and β-glucanase were the same for Pss61 and $HrpZ_{Pss}$, suggesting that $HrpZ_{Pss}$ either is a major inducer of SAR in Pss61 or is representative of SAR inducers produced by Pss61. The differences in the induction of PR proteins by *C. lagenarium* and Pss61/$HrpZ_{Pss}$ may have resulted from different inducers produced by *C. lagenarium* and Pss61/$HrpZ_{Pss}$, respectively. Alternatively, the differences may reflect possible mechan-

istic differences of plant cell death resulting from the HR caused by Pss61 or HrpZ$_{Pss}$ and disease necrosis caused by *C. lagenarium*, respectively, although both types of cell death efficiently trigger SAR in cucumber.

In this study, 80–160 μg ml^{-1} purified HrpZ$_{Pss}$ were used for induction of SAR. HrpZ$_{Pss}$ at these concentrations consistently elicited both HR and SAR in cucumber and tobacco leaves. It is not known whether these concentrations are comparable to the *in vivo* amounts of HrpZ$_{Pss}$ secreted by Pss61. Nor is it known whether the relative activity of purified HrpZ$_{Pss}$ is comparable to that of HrpZ$_{Pss}$ produced by Pss61 *in planta*. Previously, it was shown that Pss61 *hrpZ* mutants carrying transposon-induced mutations in the *hrpZ* gene (complementation group XII) were defective in the elicitation of HR (Huang *et al.*, 1991) and SAR (data not shown). More recently, it was discovered that these transposon-induced *hrpZ* mutations exert a polar effect on five downstream *hrp* genes (*hrpB–F*) in the *hrpZ* operon (Preston *et al.*, 1995; Collmer, personal communication). *hrpB–F*, like *hrpH*, are likely involved in the assembly of the Hrp secretion apparatus (Preston *et al.*, 1995). Therefore, current *hrpZ* mutations affect the expression of not only the *hrpZ* gene but also several other *hrp* genes that are involved in the secretion of HrpZ$_{Pss}$ and, most likely, other HR elicitors/pathogenicity factors. A non-polar *hrpZ* mutant is needed to assess the contribution of HrpZ$_{Pss}$ in the induction of HR and SAR. Recently, several additional proteins traversing the *P. syringae* Hrp secretion pathway have been identified in *P. syringae* pv. *tomato* (Yuan *et al.*, in preparation). It would be interesting to know whether some of these new Hrp-controlled *P. syringae* extracellular proteins can elicit HR and/or SAR.

Although the *hrpH* mutant of Pss61 failed to induce SAR in most experiments, it efficiently induced the accumulation of peroxidase and chitinase in all experiments (Figure 2b and data not shown). The induction of chitinase by *hrp* mutants was also observed by Jakobek and Lindgren (1993). These data suggest that induction of PR proteins is not necessarily a reflection of induction of SAR and that the accumulation of certain PR proteins may not contribute to resistance. In our experiments, only the accumulation of β-glucanase seemed to correlate with the SAR induced by both *C. lagenarium* and Pss61/HrpZ$_{Pss}$ in cucumber. None of the other identified PR proteins were present at high levels in systemic leaves of all cucumber plants that exhibited SAR. Whether β-glucanase is responsible for the resistance of the induced plants to *C. lagenarium*, TNV and *P. s.* pv. *lacrymans* in cucumber remains to be investigated. The relationships between the PR-A, PR-B, and PR-C proteins with β-glucanase, chitinase, or peroxidase are not known.

The demonstration of HrpZ$_{Pss}$ as a proteinaceous inducer of SAR may have important practical implications for plant disease management. Crop plants could be genetically engineered with genes encoding proteinaceous HR/SAR inducers, such as HrpZ$_{Pss}$, under the control of plant promoters inducible by virulent pathogens. If this approach were successful, the HR and SAR would be triggered in otherwise compatible interactions, limiting the disease development.

Experimental procedures

Growth of plants

Cucumber (*Cucumis sativus* L.) plants were grown in plastic pots containing Promix soil. A liquid fertilizer (Peter's 15-16-17, W. R. Grace and Co., Fogelsville, PA), containing 110 p.p.m. nitrogen, was supplied to the water, beginning when the first true leaf was fully open. Plants were grown in a glass greenhouse equipped with high-pressure sodium lights (with a photoperiod of 14 h) to supplement sunlight when necessary.

Preparation of inocula

HrpZ$_{Pss}$ was purified by affinity chromatography from *Escherichia coli* DH5α(pSYH45). pSYH45 is a derivative of pQE30 (Qiagen, Inc.) expressing a hexahistidine–HrpZ$_{Pss}$ (full-length) fusion protein. The first methionine residue of HrpZ$_{Pss}$ was replaced by the following amino acid sequence in the fusion protein: MRGSHHHHHH. The fusion protein was purified according to the manufacturer's instructions. Imidazole (300 mm) was used to elute HrpZ$_{Pss}$ protein, followed by extensive dialysis (3000-fold) in 5 mM $MgCl_2$ at 4°C. The purity of HrpZ$_{Pss}$ fusion protein was estimated by SDS–PAGE analysis to be greater than 95%. The fusion protein at the concentration of 80 μg ml^{-1} elicited a strong HR in tobacco and cucumber leaves, while an identical preparation from DH5α(pQE30) (used as a control in the purification) did not elicit any visible response in the same leaves.

Pseudomonas syringae strains were grown in King's B broth (King *et al.*, 1954) overnight at 30°C. Bacterial suspensions were prepared in 5 mM $MgSO_4$. Spores of *Colletotrichum lagenarium* were prepared as described previously (Kúc and Richmond, 1977). Tobacco necrosis virus inoculum was prepared by grinding cucumber leaves bearing necrotic local lesions in water (1g infected leaf tissue per 10 ml distilled water).

Induction of SAR

First true leaves (Leaf 1) of young cucumber plants (cv. 'Marketer') were treated with test agents by infiltration through their abaxial surfaces at 30 sites per leaf, with 10 μl per site delivered by a repeating pipettor. Treatments consisted of buffer (5 mM $MgSO_4$), HrpZ$_{Pss}$ (final concentration in buffer was 80–160 μg ml^{-1}), Pss61 or *hrpH* (a final OD_{600}=0.2 in 5 mM $MgSO_4$, equivalent to approximately 2×10^8 cells ml^{-1}), or a spore suspension of *C. lagenarium* (7.5×10^4 spores ml^{-1}).

For experiments involving tobacco (*Nicotiana tabacum* Samsun NN) plants, the third and fourth true leaves of 6-week-old plants were inoculated with TMV (100–150 lesions per leaf) or infiltrated with 120 μg ml^{-1} HrpZ$_{Pss}$ or 5 mM $MgSO_4$. For TMV inoculation, adaxial leaf surfaces were dusted with carborundum and then rubbed with a cheesecloth pad moistened with a TMV suspension. For inoculati n with HrpZ$_{Pss}$ or 5 mM $MgSO_4$, 50 μl solution was

pressured into each of 10 panels of a tobacco leaf using a needleless syringe. Five plants were used for each treatment.

Assessment of SAR

At 7–8 days after treatment of Leaf 1 with test agents, subsequently developed leaves (usually Leaf 2 and/or Leaf 3) were challenged with *C. lagenarium*, TNV or *P. s.* pv. *lacrymans*.

For fungal challenge, 20 sites per leaf received 10 μl droplets of a *C. lagenarium* spore suspension (1×10^5 spores ml^{-1}) placed on adaxial surfaces with a repeating pipettor. After inoculation, plants were held in darkened moist chambers for 24 h to facilitate penetration of leaves by the pathogen. Chambers were then gradually opened to allow plant adaptation to ambient conditions over a 12 h period, and plants were then returned to a greenhouse bench for an additional 6–7 days to allow disease development.

For TNV challenge, adaxial leaf surfaces were dusted with carborundum and then rubbed with a cheesecloth pad moistened with a TNV suspension. Virus-inoculated plants were maintained on a greenhouse bench for 8–10 days to permit disease development.

For assessment of SAR to the angular leaf spot bacterium, *P. s.* pv. *lacrymans*, Leaf 1 was infiltrated with buffer, *C. lagenarium*, or $HrpZ_{Pss}$ as described above, and Leaf 5 was challenged on the abaxial surface with the bacterium by spraying with a bacterial suspension (OD_{600}=0.1) containing 0.02% Silwet L-77, a surfactant, at 11 days post-induction or by rubbing with a cheesecloth pad saturated with a bacterial suspension (OD_{600}=0.2) at 17 days after induction treatment. Spray-inoculated leaves were misted once and plants were then placed in a darkened moist chamber for 18 h, followed by a 12 h acclimation period. Plants were subsequently returned to the greenhouse bench. Rub-inoculated leaves were misted once with water and plants were kept on a greenhouse bench. Disease was allowed to develop for 7 days for rub-inoculated plants or 13 days for spray-inoculated plants.

For evaluation of anthracnose development, the number and diameter of necrotic lesions caused by *C. lagenarium* were determined, and the total necrotic area per leaf was calculated. The extent of disease caused by TNV or *P. s.* pv. *lacrymans* was evaluated by counting necrotic local lesions on entire inoculated leaves.

For assessment of SAR to TMV, the seventh and eighth true leaves were challenge-inoculated with TMV (100–150 lesions per leaf) 5 days after induction. For each treatment the diameters of 100 TMV lesions (from 10 leaves of five plants) were recorded.

PR protein assay

Tissues were collected from Leaf 1 and Leaf 2 during the 14 day period following induction of Leaf 1. The leaf tissues were rapidly frozen with dry ice and stored at −80°C. Protein extraction was based on the method previously described (Ji and Kuc, 1995). Frozen leaf tissues were homogenized at 0–4°C in 0.1 M sodium citrate buffer, pH 5.4, containing 0.1% (v/v) β-mercaptoethanol and 0.1% (w/v) L-ascorbic acid. The homogenate was centrifuged at 12 000 *g* for 30 min. The supernatant was decanted and dialyzed against two changes of water for 24 h and then against two changes of 0.05 M sodium acetate buffer (pH 5.0) for 2 h. The extract was centrifuged again at 10 000 *g* for 10 min. The supernatant was used as crude enzyme extract. Protein concentrations were measured using the Bio-Rad protein assay kit with bovine gamma globulin as standard.

Determination of enzyme activities in cucumber leaves

Protein patterns and peroxidase isozymes were analyzed after a single separation using a 15% (w/v) native-PAGE gel (Pan *et al.*, 1989). Peroxidase activity was determined using guaiacol as substrate (Hammerschmidt *et al.*, 1982). β-1,3-glucanase and chitinase activities were detected as described elsewhere (Ji and Kuc, 1995).

Expression of pr-1 *gene in tobacco leaves*

An internal fragment (from nt 304 to 535) of the tobacco *pr-1* gene (Figure 1 in Cornelissen *et al.*, 1986) was amplified in a polymerase chain reaction (PCR) and labeled with [α-^{32}P]-dATP. Total RNA was purified from systemic leaves (the ninth true leaves) of tobacco plants 5 days post-induction. Ten micrograms of RNA from each treatment were fractionated in a 1.2% agarose/formaldehyde gel and subsequently blotted to Immobilon-N membrane (Millipore). Hybridization was performed in a solution consisting of 6×SSC, 2×Denhardt's reagent, 0.1% SDS and 10% dextran sulfate at 55°C. Washes were carried out in 0.2×SSC, 0.1% SDS at 60°C.

Acknowledgements

We wish to thank Doug Brown for growing plants, J. Shaw and G. deZoeten for providing us with TMV, David Smith, Wensheng Wei, Qian Yong, and Jing Yuan for critical review of the manuscript, and Karen Bird for help in manuscript preparation. This work was supported by grants from USDA/NRICGP (93-37303-9385) and DOE (DE-FG02-91ER20021).

References

Arlat, M., van Gijsegem, F., Huet, J.C., Pernollet, J.C. and Boucher, C.A. (1994) PopA1, a protein which induces a hypersensitivity-like response on specific *Petunia* genotypes, is secreted via the Hrp pathway of *Pseudomonas solanacearum*. *EMBO J.* 13, 543–553.

Baker, C.J., Orlandi, E.W. and Mock, N.M. (1993) Harpin, an elicitor of the hypersensitive response in tobacco caused by *Erwinia amylovora*, elicits active oxygen production in suspension cells. *Plant Physiol.* 102, 1341–1344.

Cameron, R.K., Dixon, R.A. and Lamb, C.J. (1994) Biologically induced systemic acquired resistance in *Arabidopsis thaliana*. *Plant J.* 5, 715–725.

Chen, Z., Silva, H. and Klessig, D.F. (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science*, 262, 1883–1886.

Cornelissen, B.J.C., Hooft van Huijsduijnen, R.A.M., Van Loon, L.C. and Bol, J.F. (1986) Molecular characterization of messenger RNAs for 'pathogenesis-related' proteins 1a, 1b, and 1c, induced by TMV infection of tobacco. *EMBO J.* 5, 37–40.

Dietrich, R. A., Delaney, T. P., Uknes, S. J., Ward, E. R., Ryals, J. A. and Dangl, J. L. (1994) *Arabidopsis* mutants simulating disease resistance response. *Cell*, 77, 565–577.

Fenselau, S., Balbo, I. and Bonas, U. (1992) Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. *Mol. Plant–Microbe Interact.* 5, 390–396.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. *Science*, 261, 754–756.

Hammerschmidt, R., Nuckles, E.M. and Kuc, J. (1982) Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. *Physiol. Plant Pathol.* 20, 73–82.

He, S.Y., Huang, H.-C. and Collmer, A. (1993) *Pseudomonas syringae* pv. *syringae* harpin$_{Pss}$: a protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. *Cell*, 73, 1255–1266.

He, S.Y., Bauer, D.W., Beer, S.V. and Collmer, A. (1994) The hypersensitive response elicited by the *Erwinia amylovora* harpin requires active plant metabolism. *Mol. Plant-Microbe Interact.* 7, 289–292.

Huang, H.-C., Hutcheson, S.W. and Collmer, A. (1991) Characterization of the *hrp* cluster from *Pseudomonas syringae* pv. *syringae* 61 and TnphoA tagging of genes encoding exported or membrane-spanning Hrp proteins. *Mol. Plant-Microbe Interact.* 4, 469–476.

Huang, H.-C., He, S.Y., Bauer, D.W. and Collmer, A. (1992) The *Pseudomonas syringae* pv. *syringae* 61 *hrpH* product: an envelope protein required for elicitation of the hypersensitive response in plants. *J. Bacteriol.* 174, 6878–6885.

Huang, H.-C., Lin, R.-H., Chang, C.-J., Collmer, A. and Deng, W.-L. (1995) The complete *hrp* gene cluster of *Pseudomonas syringae* pv. *syringae* 61 includes two blocks of genes required for harpin$_{Pss}$ secretion that are arranged colinearly with *Yersinia ysc* homologs. *Mol. Plant-Microbe Interact.* 8, 733–746.

Jakobek, J.L. and Lindgren, P.B. (1993) Generalized induction of defense responses in bean is not correlated with the induction of the hypersensitive reaction. *Plant Cell*, 5, 49–56.

Ji, C. and Kuc, J. (1995) Purification and characterization of an acidic β-1,3 -glucanase from cucumber and its relationship to systemic induced disease resistance induced by *Colletotrichum lagenarium* and tobacco necrosis virus. *Mol. Plant-Microbe Interact.* 8, 899–905.

Keen, N. T., Ersek, T., Long, M., Brugger, B. and Holliday, M. (1981) Inhibition of the hypersensitive response of soybean leaves to incompatible *Pseudomonas* spp. by blasticidin S, streptomycin and elevated temperature. *Physiol. Plant Pathol.* 18, 325–337.

King, E.O., Ward, M.K. and Raney, D.E. (1954) Two simple media for the demonstration of phycocyanin and fluorescin. *J. Lab. Clin. Med.* 44, 301–307.

Klement, Z. (1982) Hypersensitivity. In *Phytopathogenic Prokaryotes*, Volume 2 (Mount, M.S. and Lacy, G.H., eds). New York: Academic Press, pp. 149–177.

Kuc, J. (1982) Induced immunity to plant disease. *BioScience*, 32, 854–860.

Kuc, J. and Richmond, S. (1977) Aspects of the protection of cucumber against *Colletotrichum lagenarium* by *Colletotrichum lagenarium*. *Phytopathology*, 67, 533–536.

Malamy, J., Carr, J.P., Klessig, D.F. and Raskin, I. (1990) Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infecti n. *Science*, 250, 1002–1004

Metraux, J.-P., Signer, H., Ryals, L., Wand, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W. and Invendi, B. (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science*, 250, 1004–1006.

Metraux, J.-P., Ahl-Goy, P., Staub, T., Speich, J., Steinemann, A., Ryals, J. and Ward, E. (1991) Induced resistance in cucumber in response to 2,6-dichloroisonicotinic acid and pathogens. In *Advances in Molecular Genetics of Plant-Microbe Interactions*, Volume 6 (Hennecke, H. and Verma, D.P.S., eds). Dordrecht, Kluwer Academic Publishers, pp. 432–439.

Pan, S.Q., Ye, X.S. and Kuc, J. (1989) Direct detection of β-1,3-glucanase isozymes on polyacrylamide electrophoresis and isoelectrofocusing gels. *Anal. Biochem.* 182, 136–140.

Preston, G., Huang, H.-C., He, S.Y. and Collmer, A. (1995) The HrpZ proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea*, and *tomato* are encoded by an operon containing *Yersinia ysc* homologs and elicit the hypersensitive response in tomato but not in soybean. *Mol. Plant-Microbe Interact.* 8, 717–732.

Rasmussen, J.B., Hammerschmidt, R. and Zook, M.N. (1991) Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv. *syringae*. *Plant Physiol.* 97, 1342–1347.

Ross, A.F. (1961). Systemic acquired resistance induced by localized virus infections in plants. *Virology*, 14, 340–358.

Sequeira, L. (1979) Bacterial hypersensitivity. *Nicotiana*: procedure for experimental use. *USDA Tech. Bull.* 1586, 111–120.

Smith, A. S., Hammerschmidt, R. and Fulbright, D.W. (1991) Rapid induction of systemic resistance in cucumber by *Pseudomonas syringae* pv. *syringae*. *Physiol. Mol. Plant Pathol.* 38, 223–235.

Uknes, S., Winter, A.M., Delaney, T., Vernooij, B., Morse, A., Friedrich, L., Nye, G., Potter, S., Ward, E. and Ryals, J. (1993) Biological induction of systemic acquired resistance in *Arabidopsis*. *Mol. Plant-Microbe Interact.* 6, 692–698.

Van Gijsegem, F., Gough, C., Zischek, C., Niqueux, E., Arlat, M., Genin, S., Barberis, P., German, S., Castello, P. and Boucher, C. (1995) The *hrp* locus of *Pseudomonas solanacearum*, which controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex. *Mol. Microbiol.* 15, 1095–1114.

Wei, Z.-M. and Beer, S.V. (1993) HrpI of *Erwinia amylovora* functions in secretion of harpin and is a member of a new protein family. *J. Bacteriol.* 175, 7958–7967.

Wei, Z.-M., Laby, R.J., Zumoff, C.H., Bauer, D.W., He, S.Y., Collmer, A. and Beer, S. V. (1992) Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science*, 257, 85–88.

White, R. F. (1979) Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. *Virology*, 99, 410–412.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/82, 15/31, A01N 63/02 | A2

(11) International Publication Number: WO 00/28055

(43) International Publication Date: 18 May 2000 (18.05.00)

(21) International Application Number: PCT/US99/26039

(22) International Filing Date: 4 November 1999 (04.11.99)

(30) Priority Data:
60/107,243 5 November 1998 (05.11.98) US

(71) Applicant: EDEN BIOSCIENCE CORPORATION [US/US]; 11816 North Creek Parkway N., Bothell, WA 98011-8205 (US).

(72) Inventors: WEI, Zhong-Min; 8230 125th Court, Kirkland, WA 98034 (US). SCHADING, Richard, L.; 3607 Carriage Gate Drive, West Melbourne, FL 32904 (US).

(74) Agents: GOLDMAN, Michael, L. et al.; Nixon Peabody LLP, Clinton Square, P.O. Box 1051, Rochester, NY 14603 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: HYPERSENSITIVE RESPONSE ELICITOR-INDUCED STRESS RESISTANCE

(57) Abstract

The present invention is directed to imparting stress resistance to plants. This can be achieved by applying a hypersensitive response elicitor in a non-infectious form to plants or plant seeds under conditions effective to impart stress resistance to plants or plants grown from the plant seeds. Alternatively, transgenic plants or plant seeds transformed with a DNA molecule encoding the elicitor can be provided and the transgenic plants or plants resulting from the transgenic plant seeds are grown under conditions effective to impart stress resistance to plants or plants grown from the plant seeds.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

HYPERSENSITIVE RESPONSE ELICITOR-INDUCED STRESS RESISTANCE

FIELD OF THE INVENTION

The present invention relates to imparting stress resistance to plants with a hypersensitive response elicitor.

BACKGROUND OF THE INVENTION

Under both natural and agricultural conditions, plants are exposed to various forms of environmental stress. Stress is mainly measured with respect to growth (i.e. biomass accumulation) or with respect to the primary assimilation processes (i.e. carbon dioxide and mineral intake). Soil water deficits, suboptimal and supraoptimal temperatures, salinity, and poor aeration of soils may each cause some growth restrictions during the growing season, so that the yield of plants at the end of the season expresses only a small fraction of their genetic potential. Indeed, it is estimated that in the United States the yield of field-grown crops is only 22% of genetic potential. The same physicochemical factors can become extreme in some habitats, such as deserts or marshes, and only specially adapted vegetation can complete its life cycle in the unusually hostile conditions. In less extreme environments, individual plants can become acclimated to changes in water potential, temperature, salinity, and oxygen deficiency so that their fitness for those environments improves. Some species are better able to adapt than others, and various anatomical, structural, and biochemical mechanisms account for acclimation.

Under natural and agriculture conditions, plants must constantly endure stress. Some environmental factors can become stressful in a very short period of time (e.g., high or low temperature) or may take long periods of time to stress plants (e.g., soil water content or mineral nutrients). Generally, environmental stress effecting plants can be in the form of climatc related stress, air pollution stress,

chemical stress, and nutritional stress. Examples of climate related stress include drought, water, frost, cold temperature, high temperature, excessive light, and insufficient light. Air pollution stress can be in the form of carbon dioxide, carbon monoxide, sulfur dioxide, NO_X, hydrocarbons, ozone, ultraviolet radiation, and acidic rain. Chemical stress can result from application of insecticides, fungicides, herbicides, and heavy metals. Nutritional stress can be caused by fertilizers, micronutrients, and macronutrients.

For most plants, water is essential for growth. Some plants are able to preserve some water in the soil for later use, while others complete their life cycles during a wet season before the onset of any drought. Other plants are able to aggressively consume water to save themselves while causing water deprivation for other plants in that location. Plants lacking any of these capabilities are severely hampered by the absence of water.

Chilling injury occurs in sensitive species at temperatures that are too low for normal growth but not sufficiently low to form ice. Such injury typically occurs in species of tropical or subtropical origin. When chilling occurs, discoloration or lesions appear on leaves giving them a water-soaked appearance. If roots are chilled, the plants may wilt. On the other hand, freezing temperatures and the accompanying formation of ice crystals in plants can be lethal if ice crystals extend into protoplasts or remain for long periods.

Stress is also caused by the other temperature extremes with few plants being able to survive high temperatures. When higher plant cells or tissues are dehydrated or are not growing, they can survive higher temperatures than cells which are hydrated, vegetative, and growing. Tissues which are actively growing can rarely survive at temperatures above 45°C.

High salt concentrations are another form of environmental stress which can afflict plants. In natural conditions, such high concentrations of salt are found close to seashores and estuaries. Farther inland, natural salt may seep from geological deposits adjoining agricultural areas. In addition, salt can accumulate in irrigation water when pure water is evaporated or transpired from soil. About 1/3 of all irrigated farmland is effected by high salt concentrations. High salt content not

only injures plants but degrades soil structure by decreasing porosity and water permeability.

Air pollution in the form of ozone, carbon dioxide, carbon monoxide, sulfur dioxide, NO_x, and hydrocarbons can very adversely effect plant growth by creating smog and environmental warming.

The present invention is directed to overcoming various forms of environmental stress and imparting resistance in plants to such stress.

SUMMARY OF THE INVENTION

The present invention relates to the use of a hypersensitive response elicitor protein or polypeptide to impart stress resistance to plants. In one embodiment of the present invention, the hypersensitive response elicitor protein or polypeptide is applied to plants or plant seeds under conditions effective to impart stress resistance. Alternatively, stress resistance is imparted by providing a transgenic plant or plant seed transformed with a DNA molecule which encodes for a hypersensitive response elicitor protein or polypeptide and growing the transgenic plant or plants produced from the transgenic plant seeds under conditions effective to impart stress resistance.

Stress encompasses any environmental factor having an adverse effect on plant physiology and development. Examples of such environmental stress include climate-related stress (e.g., drought, water, frost, cold temperature, high temperature, excessive light, and insufficient light), air polllution stress (e.g., carbon dioxide, carbon monoxide, sulfur dioxide, NO_x, hydrocarbons, ozone, ultraviolet radiation, acidic rain), chemical (e.g., insecticides, fungicides, herbicides, heavy metals), and nutritional stress (e.g., fertilizer, micronutrients, macronutrients). Applicants have found that use of hypersensitive response elicitors in accordance with the present invention impart resistance to plants against such forms of environmental stress.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of a hypersensitive response elicitor protein or polypeptide to impart stress resistance to plants. In one

embodiment of the present invention, the hypersensitive response elicitor protein or polypeptide is applied to plants or plant seeds under conditions effective to impart stress resistance. Alternatively, the stress resistance is imparted by providing a transgenic plant or plant seed transformed with a DNA molecule which encodes for a hypersensitive response elicitor protein or polypeptide and growing the transgenic plant or plants produced from the transgenic plant seeds under conditions effective to impart stress resistance.

The hypersensitive response elicitor polypeptides or proteins according to the present invention are derived from hypersensitive response elicitor polypeptides or proteins of a wide variety of fungal and bacterial pathogens. Such polypeptides or proteins are able to elicit local necrosis in plant tissue contacted by the elicitor. Examples of suitable bacterial sources of polypeptide or protein elicitors include *Erwinia*, *Pseudomonas*, and *Xanthamonas* species (e.g., the following bacteria: *Erwinia amylovora*, *Erwinia chrysanthemi*, *Erwinia stewartii*, *Erwinia carotovora*, *Pseudomonas syringae*, *Pseudomonas solancearum*, *Xanthomonas campestris*, and mixtures thereof). In addition to hypersensitive response elicitors from these Gram negative bacteria, it is possible to use elicitors from Gram positive bacteria. One example is *Clavibacter michiganensis* subsp. *sepedonicus*.

An example of a fungal source of a hypersensitive response elicitor protein or polypeptide is *Phytophthora*. Suitable species of *Phytophthora* include *Phytophthora parasitica*, *Phytophthora cryptogea*, *Phytophthora cinnamomi*, *Phytophthora capsici*, *Phytophthora megasperma*, and *Phytophthora citrophthora*.

The hypersensitive response elicitor polypeptide or protein from *Erwinia chrysanthemi* has an amino acid sequence corresponding to SEQ. ID. No. 1 as follows:

```
Met Gln Ile Thr Ile Lys Ala His Ile Gly Gly Asp Leu Gly Val Ser
1               5                   10                  15

Gly Leu Gly Ala Gln Gly Leu Lys Gly Leu Asn Ser Ala Ala Ser Ser
            20                  25                  30

Leu Gly Ser Ser Val Asp Lys Leu Ser Ser Thr Ile Asp Lys Leu Thr
        35                  40                  45

Ser Ala Leu Thr Ser Met Met Phe Gly Gly Ala Leu Ala Gln Gly Leu
    50                  55                  60
```

```
Gly Ala Ser Ser Lys Gly Leu Gly Met Ser Asn Gln Leu Gly Gln Ser
65                  70                  75                  80

Phe Gly Asn Gly Ala Gln Gly Ala Ser Asn Leu Leu Ser Val Pro Lys
                85                  90                  95

Ser Gly Gly Asp Ala Leu Ser Lys Met Phe Asp Lys Ala Leu Asp Asp
            100                 105                 110

Leu Leu Gly His Asp Thr Val Thr Lys Leu Thr Asn Gln Ser Asn Gln
        115                 120                 125

Leu Ala Asn Ser Met Leu Asn Ala Ser Gln Met Thr Gln Gly Asn Met
    130                 135                 140

Asn Ala Phe Gly Ser Gly Val Asn Asn Ala Leu Ser Ser Ile Leu Gly
145                 150                 155                 160

Asn Gly Leu Gly Gln Ser Met Ser Gly Phe Ser Gln Pro Ser Leu Gly
                165                 170                 175

Ala Gly Gly Leu Gln Gly Leu Ser Gly Ala Gly Ala Phe Asn Gln Leu
            180                 185                 190

Gly Asn Ala Ile Gly Met Gly Val Gly Gln Asn Ala Ala Leu Ser Ala
        195                 200                 205

Leu Ser Asn Val Ser Thr His Val Asp Gly Asn Asn Arg His Phe Val
    210                 215                 220

Asp Lys Glu Asp Arg Gly Met Ala Lys Glu Ile Gly Gln Phe Met Asp
225                 230                 235                 240

Gln Tyr Pro Glu Ile Phe Gly Lys Pro Glu Tyr Gln Lys Asp Gly Trp
                245                 250                 255

Ser Ser Pro Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser Lys
            260                 265                 270

Pro Asp Asp Asp Gly Met Thr Gly Ala Ser Met Asp Lys Phe Arg Gln
        275                 280                 285

Ala Met Gly Met Ile Lys Ser Ala Val Ala Gly Asp Thr Gly Asn Thr
    290                 295                 300

Asn Leu Asn Leu Arg Gly Ala Gly Gly Ala Ser Leu Gly Ile Asp Ala
305                 310                 315                 320

Ala Val Val Gly Asp Lys Ile Ala Asn Met Ser Leu Gly Lys Leu Ala
                325                 330                 335

Asn Ala
```

This hypersensitive response elicitor polypeptide or protein has a molecular weight of 34 kDa, is heat stable, has a glycine content of greater than 16%, and contains

substantially no cysteine. The *Erwinia chrysanthemi* hypersensitive response elicitor polypeptide or protein is encoded by a DNA molecule having a nucleotide sequence corresponding to SEQ. ID. No. 2 as follows:

```
CGATTTTACC CGGGTGAACG TGCTATGACC GACAGCATCA CGGTATTCGA CACCGTTACG      60
GCGTTTATGG CCGCGATGAA CCGGCATCAG GCGGCGCGCT GGTCGCCGCA ATCCGGCGTC     120
GATCTGGTAT TTCAGTTTGG GGACACCGGG CGTGAACTCA TGATGCAGAT TCAGCCGGGG     180
CAGCAATATC CCGGCATGTT GCGCACGCTG CTCGCTCGTC GTTATCAGCA GGCGGCAGAG     240
TGCGATGGCT GCCATCTGTG CCTGAACGGC AGCGATGTAT TGATCCTCTG GTGGCCGCTG     300
CCGTCGGATC CCGGCAGTTA TCCGCAGGTG ATCGAACGTT TGTTTGAACT GGCGGGAATG     360
ACGTTGCCGT CGCTATCCAT AGCACCGACG GCGCGTCCGC AGACAGGGAA CGGACGCGCC     420
CGATCATTAA GATAAAGGCG GCTTTTTTTA TTGCAAAACG GTAACGGTGA GGAACCGTTT     480
CACCGTCGGC GTCACTCAGT AACAAGTATC CATCATGATG CCTACATCGG GATCGGCGTG     540
GGCATCCGTT GCAGATACTT TTGCGAACAC CTGACATGAA TGAGGAAACG AAATTATGCA     600
AATTACGATC AAAGCGCACA TCGGCGGTGA TTTGGGCGTC TCCGGTCTGG GCTGGGTGC      660
TCAGGGACTG AAAGGACTGA ATTCCGCGGC TTCATCGCTG GGTTCCAGCG TGGATAAACT     720
GAGCAGCACC ATCGATAAGT TGACCTCCGC GCTGACTTCG ATGATGTTTG GCGGCGCGCT     780
GGCGCAGGGG CTGGGCGCCA GCTCGAAGGG GCTGGGGATG AGCAATCAAC TGGGCCAGTC     840
TTTCGGCAAT GGCGCGCAGG GTGCGAGCAA CCTGCTATCC GTACCGAAAT CCGGCGGCGA     900
TGCGTTGTCA AAAATGTTTG ATAAAGCGCT GGACGATCTG CTGGGTCATG ACACCGTGAC     960
CAAGCTGACT AACCAGAGCA ACCAACTGGC TAATTCAATG CTGAACGCCA GCCAGATGAC    1020
CCAGGGTAAT ATGAATGCGT TCGGCAGCGG TGTGAACAAC GCACTGTCGT CCATTCTCGG    1080
CAACGGTCTC GGCCAGTCGA TGAGTGGCTT CTCTCAGCCT TCTCTGGGGG CAGGCGGCTT    1140
GCAGGGCCTG AGCGGCGCGG GTGCATTCAA CCAGTTGGGT AATGCCATCG GCATGGGCGT    1200
GGGGCAGAAT GCTGCGCTGA GTGCGTTGAG TAACGTCAGC ACCCACGTAG ACGGTAACAA    1260
CCGCCACTTT GTAGATAAAG AAGATCGCGG CATGGCGAAA GAGATCGGCC AGTTTATGGA    1320
TCAGTATCCG GAAATATTCG GTAAACCGGA ATACCAGAAA GATGGCTGGA GTTCGCCGAA    1380
GACGGACGAC AAATCCTGGG CTAAAGCGCT GAGTAAACCG GATGATGACG GTATGACCGG    1440
CGCCAGCATG GACAAATTCC GTCAGGCGAT GGGTATGATC AAAAGCGCGG TGGCGGGTGA    1500
TACCGGCAAT ACCAACCTGA ACCTGCGTGG CGCGGGCGGT GCATCGCTGG GTATCGATGC    1560
GGCTGTCGTC GGCGATAAAA TAGCCAACAT GTCGCTGGGT AAGCTGGCCA ACGCCTGATA    1620
```

```
ATCTGTGCTG GCCTGATAAA GCGGAAACGA AAAAAGAGAC GGGGAAGCCT GTCTCTTTTC    1680
TTATTATGCG GTTTATGCGG TTACCTGGAC CGGTTAATCA TCGTCATCGA TCTGGTACAA    1740
ACGCACATTT TCCCGTTCAT TCGCGTCGTT ACGCGCCACA ATCGCGATGG CATCTTCCTC    1800
GTCGCTCAGA TTGCGCGGCT GATGGGGAAC GCCGGGTGGA ATATAGAGAA ACTCGCCGGC    1860
CAGATGGAGA CACGTCTGCG ATAAATCTGT GCCGTAACGT GTTTCTATCC GCCCCTTTAG    1920
CAGATAGATT GCGGTTTCGT AATCAACATG GTAATGCGGT TCCGCCTGTG CGCCGGCCGG    1980
GATCACCACA ATATTCATAG AAAGCTGTCT TGCACCTACC GTATCGCGGG AGATACCGAC    2040
AAAATAGGGC AGTTTTTGCG TGGTATCCGT GGGGTGTTCC GGCCTGACAA TCTTGAGTTG    2100
GTTCGTCATC ATCTTTCTCC ATCTGGGCGA CCTGATCGGT T                        2141
```

The hypersensitive response elicitor polypeptide or protein derived from *Erwinia amylovora* has an amino acid sequence corresponding to SEQ. ID. No. 3 as follows:

```
Met Ser Leu Asn Thr Ser Gly Leu Gly Ala Ser Thr Met Gln Ile Ser
1               5                   10                  15

Ile Gly Gly Ala Gly Gly Asn Asn Gly Leu Leu Gly Thr Ser Arg Gln
            20                  25                  30

Asn Ala Gly Leu Gly Gly Asn Ser Ala Leu Gly Leu Gly Gly Gly Asn
        35                  40                  45

Gln Asn Asp Thr Val Asn Gln Leu Ala Gly Leu Leu Thr Gly Met Met
    50                  55                  60

Met Met Met Ser Met Met Gly Gly Gly Gly Leu Met Gly Gly Gly Leu
65                  70                  75                  80

Gly Gly Gly Leu Gly Asn Gly Leu Gly Gly Ser Gly Gly Leu Gly Glu
                85                  90                  95

Gly Leu Ser Asn Ala Leu Asn Asp Met Leu Gly Gly Ser Leu Asn Thr
            100                 105                 110

Leu Gly Ser Lys Gly Gly Asn Asn Thr Thr Ser Thr Thr Asn Ser Pro
        115                 120                 125

Leu Asp Gln Ala Leu Gly Ile Asn Ser Thr Ser Gln Asn Asp Asp Ser
    130                 135                 140

Thr Ser Gly Thr Asp Ser Thr Ser Asp Ser Ser Asp Pro Met Gln Gln
145                 150                 155                 160

Leu Leu Lys Met Phe Ser Glu Ile Met Gln Ser Leu Phe Gly Asp Gly
                165                 170                 175
```

```
Gln Asp Gly Thr Gln Gly Ser Ser Ser Gly Gly Lys Gln Pro Thr Glu
            180                 185                 190

Gly Glu Gln Asn Ala Tyr Lys Lys Gly Val Thr Asp Ala Leu Ser Gly
        195                 200                 205

Leu Met Gly Asn Gly Leu Ser Gln Leu Leu Gly Asn Gly Gly Leu Gly
    210                 215                 220

Gly Gly Gln Gly Gly Asn Ala Gly Thr Gly Leu Asp Gly Ser Ser Leu
225                 230                 235                 240

Gly Gly Lys Gly Leu Gln Asn Leu Ser Gly Pro Val Asp Tyr Gln Gln
                245                 250                 255

Leu Gly Asn Ala Val Gly Thr Gly Ile Gly Met Lys Ala Gly Ile Gln
            260                 265                 270

Ala Leu Asn Asp Ile Gly Thr His Arg His Ser Ser Thr Arg Ser Phe
        275                 280                 285

Val Asn Lys Gly Asp Arg Ala Met Ala Lys Glu Ile Gly Gln Phe Met
    290                 295                 300

Asp Gln Tyr Pro Glu Val Phe Gly Lys Pro Gln Tyr Gln Lys Gly Pro
305                 310                 315                 320

Gly Gln Glu Val Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser
                325                 330                 335

Lys Pro Asp Asp Asp Gly Met Thr Pro Ala Ser Met Glu Gln Phe Asn
            340                 345                 350

Lys Ala Lys Gly Met Ile Lys Arg Pro Met Ala Gly Asp Thr Gly Asn
        355                 360                 365

Gly Asn Leu Gln Ala Arg Gly Ala Gly Gly Ser Ser Leu Gly Ile Asp
    370                 375                 380

Ala Met Met Ala Gly Asp Ala Ile Asn Asn Met Ala Leu Gly Lys Leu
385                 390                 395                 400

Gly Ala Ala
```

This hypersensitive response elicitor polypeptide or protein has a molecular weight of about 39 kDa, has a pI of approximately 4.3, and is heat stable at 100°C for at least 10 minutes. This hypersensitive response elicitor polypeptide or protein has substantially no cysteine. The hypersensitive response elicitor polypeptide or protein derived from *Erwinia amylovora* is more fully described in Wei, Z.-M., R. J. Laby, C. H. Zumoff, D. W. Bauer, S.-Y. He, A. Collmer, and S. V. Beer, "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*,"

Science 257:85-88 (1992), which is hereby incorporated by reference. The DNA molecule encoding this polypeptide or protein has a nucleotide sequence corresponding to SEQ. ID. No. 4 as follows:

```
AAGCTTCGGC ATGGCACGTT TGACCGTTGG GTCGGCAGGG TACGTTTGAA TTATTCATAA     60
GAGGAATACG TTATGAGTCT GAATACAAGT GGGCTGGGAG CGTCAACGAT GCAAATTTCT    120
ATCGGCGGTG CGGGCGGAAA TAACGGGTTG CTGGGTACCA GTCGCCAGAA TGCTGGGTTG    180
GGTGGCAATT CTGCACTGGG GCTGGGCGGC GGTAATCAAA ATGATACCGT CAATCAGCTG    240
GCTGGCTTAC TCACCGGCAT GATGATGATG ATGAGCATGA TGGGCGGTGG TGGGCTGATG    300
GGCGGTGGCT TAGGCGGTGG CTTAGGTAAT GGCTTGGGTG GCTCAGGTGG CCTGGGCGAA    360
GGACTGTCGA ACGCGCTGAA CGATATGTTA GGCGGTTCGC TGAACACGCT GGGCTCGAAA    420
GGCGGCAACA ATACCACTTC AACAACAAAT TCCCCGCTGG ACCAGGCGCT GGGTATTAAC    480
TCAACGTCCC AAAACGACGA TTCCACCTCC GGCACAGATT CCACCTCAGA CTCCAGCGAC    540
CCGATGCAGC AGCTGCTGAA GATGTTCAGC GAGATAATGC AAAGCCTGTT TGGTGATGGG    600
CAAGATGGCA CCCAGGGCAG TTCCTCTGGG GGCAAGCAGC CGACCGAAGG CGAGCAGAAC    660
GCCTATAAAA AAGGAGTCAC TGATGCGCTG TCGGGCCTGA TGGGTAATGG TCTGAGCCAG    720
CTCCTTGGCA ACGGGGGACT GGGAGGTGGT CAGGGCGGTA ATGCTGGCAC GGGTCTTGAC    780
GGTTCGTCGC TGGGCGGCAA AGGGCTGCAA AACCTGAGCG GGCCGGTGGA CTACCAGCAG    840
TTAGGTAACG CCGTGGGTAC CGGTATCGGT ATGAAAGCGG GCATTCAGGC GCTGAATGAT    900
ATCGGTACGC ACAGGCACAG TTCAACCCGT TCTTTCGTCA ATAAAGGCGA TCGGGCGATG    960
GCGAAGGAAA TCGGTCAGTT CATGGACCAG TATCCTGAGG TGTTTGGCAA GCCGCAGTAC   1020
CAGAAAGGCC CGGGTCAGGA GGTGAAAACC GATGACAAAT CATGGGCAAA AGCACTGAGC   1080
AAGCCAGATG ACGACGGAAT GACACCAGCC AGTATGGAGC AGTTCAACAA AGCCAAGGGC   1140
ATGATCAAAA GGCCCATGGC GGGTGATACC GGCAACGGCA ACCTGCAGGC ACGCGGTGCC   1200
GGTGGTTCTT CGCTGGGTAT TGATGCCATG ATGGCCGGTG ATGCCATTAA CAATATGGCA   1260
CTTGGCAAGC TGGGCGCGGC TTAAGCTT                                      1288
```

Another potentially suitable hypersensitive response elicitor from *Erwinia amylovora* is disclosed in U.S. Patent Application Serial No. 09/120,927, which is hereby incorporated by reference. The protein is encoded by a DNA molecule having a nucleic acid sequence of SEQ. ID. No. 5 as follows:

```
ATGTCAATTC TTACGCTTAA CAACAATACC TCGTCCTCGC CGGGTCTGTT CCAGTCCGGG     60
GGGGACAACG GGCTTGGTGG TCATAATGCA AATTCTGCGT TGGGGCAACA ACCCATCGAT    120
CGGCAAACCA TTGAGCAAAT GGCTCAATTA TTGGCGGAAC TGTTAAAGTC ACTGCTATCG    180
CCACAATCAG GTAATGCGGC AACCGGAGCC GGTGGCAATG ACCAGACTAC AGGAGTTGGT    240
AACGCTGGCG GCCTGAACGG ACGAAAAGGC ACAGCAGGAA CCACTCCGCA GTCTGACAGT    300
CAGAACATGC TGAGTGAGAT GGGCAACAAC GGGCTGGATC AGGCCATCAC GCCCGATGGC    360
CAGGGCGGCG GGCAGATCGG CGATAATCCT TTACTGAAAG CCATGCTGAA GCTTATTGCA    420
CGCATGATGG ACGGCCAAAG CGATCAGTTT GGCCAACCTG GTACGGGCAA CAACAGTGCC    480
TCTTCCGGTA CTTCTTCATC TGGCGGTTCC CCTTTTAACG ATCTATCAGG GGGGAAGGCC    540
CCTTCCGGCA ACTCCCCTTC CGGCAACTAC TCTCCCGTCA GTACCTTCTC ACCCCCATCC    600
ACGCCAACGT CCCCTACCTC ACCGCTTGAT TTCCCTTCTT CTCCCACCAA AGCAGCCGGG    660
GGCAGCACGC CGGTAACCGA TCATCCTGAC CCTGTTGGTA GCGCGGGCAT CGGGGCCGGA    720
AATTCGGTGG CCTTCACCAG CGCCGGCGCT AATCAGACGG TGCTGCATGA CACCATTACC    780
GTGAAAGCGG GTCAGGTGTT TGATGGCAAA GGACAAACCT TCACCGCCGG TTCAGAATTA    840
GGCGATGGCG GCCAGTCTGA AAACCAGAAA CCGCTGTTTA TACTGGAAGA CGGTGCCAGC    900
CTGAAAAACG TCACCATGGG CGACGACGGG GCGGATGGTA TTCATCTTTA CGGTGATGCC    960
AAAATAGACA ATCTGCACGT CACCAACGTG GGTGAGGACG CGATTACCGT TAAGCCAAAC   1020
AGCGCGGGCA AAAAATCCCA CGTTGAAATC ACTAACAGTT CCTTCGAGCA CGCCTCTGAC   1080
AAGATCCTGC AGCTGAATGC CGATACTAAC CTGAGCGTTG ACAACGTGAA GGCCAAAGAC   1140
TTTGGTACTT TTGTACGCAC TAACGGCGGT CAACAGGGTA ACTGGGATCT GAATCTGAGC   1200
CATATCAGCG CAGAAGACGG TAAGTTCTCG TTCGTTAAAA GCGATAGCGA GGGGCTAAAC   1260
GTCAATACCA GTGATATCTC ACTGGGTGAT GTTGAAAACC ACTACAAAGT GCCGATGTCC   1320
GCCAACCTGA AGGTGGCTGA ATGA                                          1344
```

See GenBank Accession No. U94513. The isolated DNA molecule of the present invention encodes a hypersensitive response elicitor protein or polypeptide having an amino acid sequence of SEQ. ID. No. 6 as follows:

```
Met Ser Ile Leu Thr Leu Asn Asn Asn Thr Ser Ser Ser Pro Gly Leu
1               5                   10                  15

Phe Gln Ser Gly Gly Asp Asn Gly Leu Gly Gly His Asn Ala Asn Ser
            20                  25                  30

Ala Leu Gly Gln Gln Pro Ile Asp Arg Gln Thr Ile Glu Gln Met Ala
        35                  40                  45
```

```
Gln Leu Leu Ala Glu Leu Leu Lys Ser Leu Leu Ser Pro Gln Ser Gly
    50                  55                  60

Asn Ala Ala Thr Gly Ala Gly Gly Asn Asp Gln Thr Thr Gly Val Gly
65                  70                  75                  80

Asn Ala Gly Gly Leu Asn Gly Arg Lys Gly Thr Ala Gly Thr Thr Pro
                85                  90                  95

Gln Ser Asp Ser Gln Asn Met Leu Ser Glu Met Gly Asn Asn Gly Leu
            100                 105                 110

Asp Gln Ala Ile Thr Pro Asp Gly Gln Gly Gly Gly Gln Ile Gly Asp
        115                 120                 125

Asn Pro Leu Leu Lys Ala Met Leu Lys Leu Ile Ala Arg Met Met Asp
    130                 135                 140

Gly Gln Ser Asp Gln Phe Gly Gln Pro Gly Thr Gly Asn Asn Ser Ala
145                 150                 155                 160

Ser Ser Gly Thr Ser Ser Ser Gly Gly Ser Pro Phe Asn Asp Leu Ser
                165                 170                 175

Gly Gly Lys Ala Pro Ser Gly Asn Ser Pro Ser Gly Asn Tyr Ser Pro
            180                 185                 190

Val Ser Thr Phe Ser Pro Pro Ser Thr Pro Thr Ser Pro Thr Ser Pro
        195                 200                 205

Leu Asp Phe Pro Ser Ser Pro Thr Lys Ala Ala Gly Gly Ser Thr Pro
    210                 215                 220

Val Thr Asp His Pro Asp Pro Val Gly Ser Ala Gly Ile Gly Ala Gly
225                 230                 235                 240

Asn Ser Val Ala Phe Thr Ser Ala Gly Ala Asn Gln Thr Val Leu His
                245                 250                 255

Asp Thr Ile Thr Val Lys Ala Gly Gln Val Phe Asp Gly Lys Gly Gln
            260                 265                 270

Thr Phe Thr Ala Gly Ser Glu Leu Gly Asp Gly Gly Gln Ser Glu Asn
        275                 280                 285

Gln Lys Pro Leu Phe Ile Leu Glu Asp Gly Ala Ser Leu Lys Asn Val
    290                 295                 300

Thr Met Gly Asp Asp Gly Ala Asp Gly Ile His Leu Tyr Gly Asp Ala
305                 310                 315                 320

Lys Ile Asp Asn Leu His Val Thr Asn Val Gly Glu Asp Ala Ile Thr
                325                 330                 335

Val Lys Pro Asn Ser Ala Gly Lys Lys Ser His Val Glu Ile Thr Asn
            340                 345                 350
```

```
Ser Ser Phe Glu His Ala Ser Asp Lys Ile Leu Gln Leu Asn Ala Asp
        355                 360                 365

Thr Asn Leu Ser Val Asp Asn Val Lys Ala Lys Asp Phe Gly Thr Phe
    370                 375                 380

Val Arg Thr Asn Gly Gly Gln Gln Gly Asn Trp Asp Leu Asn Leu Ser
385                 390                 395                 400

His Ile Ser Ala Glu Asp Gly Lys Phe Ser Phe Val Lys Ser Asp Ser
                405                 410                 415

Glu Gly Leu Asn Val Asn Thr Ser Asp Ile Ser Leu Gly Asp Val Glu
            420                 425                 430

Asn His Tyr Lys Val Pro Met Ser Ala Asn Leu Lys Val Ala Glu
        435                 440                 445
```

This protein or polypeptide is acidic, rich in glycine and serine, and lacks cysteine. It is also heat stable, protease sensitive, and suppressed by inhibitors of plant metabolism. The protein or polypeptide of the present invention has a predicted molecular size of ca. 4.5 kDa.

Another potentially suitable hypersensitive response elicitor from *Erwinia amylovora* is disclosed in U.S. Patent Application Serial No. 09/120,663, which is hereby incorporated by reference. The protein is encoded by a DNA molecule having a nucleic acid sequence of SEQ. ID. No. 7 as follows:

```
ATGGAATTAA AATCACTGGG AACTGAACAC AAGGCGGCAG TACACACAGC GGCGCACAAC     60
CCTGTGGGGC ATGGTGTTGC CTTACAGCAG GGCAGCAGCA GCAGCAGCCC GCAAAATGCC    120
GCTGCATCAT TGGCGGCAGA AGGCAAAAAT CGTGGGAAAA TGCCGAGAAT TCACCAGCCA    180
TCTACTGCGG CTGATGGTAT CAGCGCTGCT CACCAGCAAA AGAAATCCTT CAGTCTCAGG    240
GGCTGTTTGG GGACGAAAAA ATTTTCCAGA TCGGCACCGC AGGGCCAGCC AGGTACCACC    300
CACAGCAAAG GGGCAACATT GCGCGATCTG CTGGCGCGGG ACGACGGCGA AACGCAGCAT    360
GAGGCGGCCG CGCCAGATGC GGCGCGTTTG ACCCGTTCGG GCGGCGTCAA ACGCCGCAAT    420
ATGGACGACA TGGCCGGGCG GCCAATGGTG AAAGGTGGCA GCGGCGAAGA TAAGGTACCA    480
ACGCAGCAAA AACGGCATCA GCTGAACAAT TTTGGCCAGA TGCGCCAAAC GATGTTGAGC    540
AAAATGGCTC ACCCGGCTTC AGCCAACGCC GGCGATCGCC TGCAGCATTC ACCGCCGCAC    600
ATCCCGGGTA GCCACCACGA AATCAAGGAA GAACCGGTTG GCTCCACCAG CAAGGCAACA    660
ACGGCCCACG CAGACAGAGT GGAAATCGCT CAGGAAGATG ACGACAGCGA ATTCCAGCAA    720
CTGCATCAAC AGCGGCTGGC GCGCGAACGG GAAAATCCAC CGCAGCCGCC CAAACTCGGC    780
GTTGCCACAC CGATTAGCGC CAGGTTTCAG CCCAAACTGA CTGCGGTTGC GGAAAGCGTC    840
```

```
CTTGAGGGGA CAGATACCAC GCAGTCACCC CTTAAGCCGC AATCAATGCT GAAAGGAAGT    900
GGAGCCGGGG TAACGCCGCT GGCGGTAACG CTGGATAAAG GCAAGTTGCA GCTGGCACCG    960
GATAATCCAC CCGCGCTCAA TACGTTGTTG AAGCAGACAT TGGGTAAAGA CACCCAGCAC   1020
TATCTGGCGC ACCATGCCAG CAGCGACGGT AGCCAGCATC TGCTGCTGGA CAACAAAGGC   1080
CACCTGTTTG ATATCAAAAG CACCGCCACC AGCTATAGCG TGCTGCACAA CAGCCACCCC   1140
GGTGAGATAA AGGGCAAGCT GGCGCAGGCG GGTACTGGCT CCGTCAGCGT AGACGGTAAA   1200
AGCGGCAAGA TCTCGCTGGG GAGCGGTACG CAAAGTCACA ACAAAACAAT GCTAAGCCAA   1260
CCGGGGGAAG CGCACCGTTC CTTATTAACC GGCATTTGGC AGCATCCTGC TGGCGCAGCG   1320
CGGCCGCAGG GCGAGTCAAT CCGCCTGCAT GACGACAAAA TTCATATCCT GCATCCGGAG   1380
CTGGGCGTAT GGCAATCTGC GGATAAAGAT ACCCACAGCC AGCTGTCTCG CCAGGCAGAC   1440
GGTAAGCTCT ATGCGCTGAA AGACAACCGT ACCCTGCAAA ACCTCTCCGA TAATAAATCC   1500
TCAGAAAAGC TGGTCGATAA AATCAAATCG TATTCCGTTG ATCAGCGGGG GCAGGTGGCG   1560
ATCCTGACGG ATACTCCCGG CCGCCATAAG ATGAGTATTA TGCCCTCGCT GGATGCTTCC   1620
CCGGAGAGCC ATATTTCCCT CAGCCTGCAT TTTGCCGATG CCCACCAGGG GTTATTGCAC   1680
GGGAAGTCGG AGCTTGAGGC ACAATCTGTC GCGATCAGCC ATGGGCGACT GGTTGTGGCC   1740
GATAGCGAAG GCAAGCTGTT TAGCGCCGCC ATTCCGAAGC AAGGGGATGG AAACGAACTG   1800
AAAATGAAAG CCATGCCTCA GCATGCGCTC GATGAACATT TTGGTCATGA CCACCAGATT   1860
TCTGGATTTT TCCATGACGA CCACGGCCAG CTTAATGCGC TGGTGAAAAA TAACTTCAGG   1920
CAGCAGCATG CCTGCCCGTT GGGTAACGAT CATCAGTTTC ACCCCGGCTG GAACCTGACT   1980
GATGCGCTGG TTATCGACAA TCAGCTGGGG CTGCATCATA CCAATCCTGA ACCGCATGAG   2040
ATTCTTGATA TGGGGCATTT AGGCAGCCTG GCGTTACAGG AGGGCAAGCT TCACTATTTT   2100
GACCAGCTGA CCAAAGGGTG GACTGGCGCG GAGTCAGATT GTAAGCAGCT GAAAAAAGGC   2160
CTGGATGGAG CAGCTTATCT ACTGAAAGAC GGTGAAGTGA AACGCCTGAA TATTAATCAG   2220
AGCACCTCCT CTATCAAGCA CGGAACGGAA AACGTTTTTT CGCTGCCGCA TGTGCGCAAT   2280
AAACCGGAGC CGGGAGATGC CCTGCAAGGG CTGAATAAAG ACGATAAGGC CCAGGCCATG   2340
GCGGTGATTG GGGTAAATAA ATACCTGGCG CTGACGGAAA AAGGGGACAT TCGCTCCTTC   2400
CAGATAAAAC CCGGCACCCA GCAGTTGGAG CGGCCGGCAC AAACTCTCAG CCGCGAAGGT   2460
ATCAGCGGCG AACTGAAAGA CATTCATGTC GACCACAAGC AGAACCTGTA TGCCTTGACC   2520
CACGAGGGAG AGGTGTTTCA TCAGCCGCGT GAAGCCTGGC AGAATGGTGC CGAAAGCAGC   2580
AGCTGGCACA AACTGGCGTT GCCACAGAGT GAAAGTAAGC TAAAAAGTCT GGACATGAGC   2640
CATGAGCACA AACCGATTGC CACCTTTGAA GACGGTAGCC AGCATCAGCT GAAGGCTGGC   2700
GGCTGGCACG CCTATGCGGC ACCTGAACGC GGGCCGCTGG CGGTGGGTAC CAGCGGTTCA   2760
```

CAAACCGTCT TTAACCGACT AATGCAGGGG GTGAAAGGCA AGGTGATCCC AGGCAGCGGG 2820

TTGACGGTTA AGCTCTCGGC TCAGACGGGG GGAATGACCG GCGCCGAAGG GCGCAAGGTC 2880

AGCAGTAAAT TTTCCGAAAG GATCCGCGCC TATGCGTTCA ACCCAACAAT GTCCACGCCG 2940

CGACCGATTA AAAATGCTGC TTATGCCACA CAGCACGGCT GGCAGGGGCG TGAGGGGTTG 3000

AAGCCGTTGT ACGAGATGCA GGGAGCGCTG ATTAAACAAC TGGATGCGCA TAACGTTCGT 3060

CATAACGCGC CACAGCCAGA TTTGCAGAGC AAACTGGAAA CTCTGGATTT AGGCGAACAT 3120

GGCGCAGAAT TGCTTAACGA CATGAAGCGC TTCCGCGACG AACTGGAGCA GAGTGCAACC 3180

CGTTCGGTGA CCGTTTTAGG TCAACATCAG GGAGTGCTAA AAAGCAACGG TGAAATCAAT 3240

AGCGAATTTA AGCCATCGCC CGGCAAGGCG TTGGTCCAGA GCTTTAACGT CAATCGCTCT 3300

GGTCAGGATC TAAGCAAGTC ACTGCAACAG GCAGTACATG CCACGCCGCC ATCCGCAGAG 3360

AGTAAACTGC AATCCATGCT GGGGCACTTT GTCAGTGCCG GGGTGGATAT GAGTCATCAG 3420

AAGGGCGAGA TCCCGCTGGG CCGCCAGCGC GATCCGAATG ATAAAACCGC ACTGACCAAA 3480

TCGCGTTTAA TTTTAGATAC CGTGACCATC GGTGAACTGC ATGAACTGGC CGATAAGGCG 3540

AAACTGGTAT CTGACCATAA ACCCGATGCC GATCAGATAA AACAGCTGCG CCAGCAGTTC 3600

GATACGCTGC GTGAAAAGCG GTATGAGAGC AATCCGGTGA AGCATTACAC CGATATGGGC 3660

TTCACCCATA ATAAGGCGCT GGAAGCAAAC TATGATGCGG TCAAAGCCTT TATCAATGCC 3720

TTTAAGAAAG AGCACCACGG CGTCAATCTG ACCACGCGTA CCGTACTGGA ATCACAGGGC 3780

AGTGCGGAGC TGGCGAAGAA GCTCAAGAAT ACGCTGTTGT CCCTGGACAG TGGTGAAAGT 3840

ATGAGCTTCA GCCGGTCATA TGGCGGGGGC GTCAGCACTG TCTTTGTGCC TACCCTTAGC 3900

AAGAAGGTGC CAGTTCCGGT GATCCCCGGA GCCGGCATCA CGCTGGATCG CGCCTATAAC 3960

CTGAGCTTCA GTCGTACCAG CGGCGGATTG AACGTCAGTT TTGGCCGCGA CGGCGGGGTG 4020

AGTGGTAACA TCATGGTCGC TACCGGCCAT GATGTGATGC CCTATATGAC CGGTAAGAAA 4080

ACCAGTGCAG GTAACGCCAG TGACTGGTTG AGCGCAAAAC ATAAAATCAG CCCGGACTTG 4140

CGTATCGGCG CTGCTGTGAG TGGCACCCTG CAAGGAACGC TACAAAACAG CCTGAAGTTT 4200

AAGCTGACAG AGGATGAGCT GCCTGGCTTT ATCCATGGCT TGACGCATGG CACGTTGACC 4260

CCGGCAGAAC TGTTGCAAAA GGGGATCGAA CATCAGATGA AGCAGGGCAG CAAACTGACG 4320

TTTAGCGTCG ATACCTCGGC AAATCTGGAT CTGCGTGCCG GTATCAATCT GAACGAAGAC 4380

GGCAGTAAAC CAAATGGTGT CACTGCCCGT GTTTCTGCCG GGCTAAGTGC ATCGGCAAAC 4440

CTGGCCGCCG GCTCGCGTGA ACGCAGCACC ACCTCTGGCC AGTTTGGCAG CACGACTTCG 4500

GCCAGCAATA ACCGCCCAAC CTTCCTCAAC GGGGTCGGCG CGGGTGCTAA CCTGACGGCT 4560

GCTTTAGGGG TTGCCCATTC ATCTACGCAT GAAGGGAAAC CGGTCGGGAT CTTCCCGGCA 4620

TTTACCTCGA CCAATGTTTC GGCAGCGCTG GCGCTGGATA ACCGTACCTC ACAGAGTATC 4680

AGCCTGGAAT TGAAGCGCGC GGAGCCGGTG ACCAGCAACG ATATCAGCGA GTTGACCTCC 4740

```
ACGCTGGGAA AACACTTTAA GGATAGCGCC ACAACGAAGA TGCTTGCCGC TCTCAAAGAG    4800
TTAGATGACG CTAAGCCCGC TGAACAACTG CATATTTTAC AGCAGCATTT CAGTGCAAAA    4860
GATGTCGTCG GTGATGAACG CTACGAGGCG GTGCGCAACC TGAAAAAACT GGTGATACGT    4920
CAACAGGCTG CGGACAGCCA CAGCATGGAA TTAGGATCTG CCAGTCACAG CACGACCTAC    4980
AATAATCTGT CGAGAATAAA TAATGACGGC ATTGTCGAGC TGCTACACAA ACATTTCGAT    5040
GCGGCATTAC CAGCAAGCAG TGCCAAACGT CTTGGTGAAA TGATGAATAA CGATCCGGCA    5100
CTGAAAGATA TTATTAAGCA GCTGCAAAGT ACGCCGTTCA GCAGCGCCAG CGTGTCGATG    5160
GAGCTGAAAG ATGGTCTGCG TGAGCAGACG GAAAAAGCAA TACTGGACGG TAAGGTCGGT    5220
CGTGAAGAAG TGGGAGTACT TTTCCAGGAT CGTAACAACT TGCGTGTTAA ATCGGTCAGC    5280
GTCAGTCAGT CCGTCAGCAA AAGCGAAGGC TTCAATACCC CAGCGCTGTT ACTGGGGACG    5340
AGCAACAGCG CTGCTATGAG CATGGAGCGC AACATCGGAA CCATTAATTT TAAATACGGC    5400
CAGGATCAGA ACACCCCACG GCGATTTACC CTGGAGGGTG GAATAGCTCA GGCTAATCCG    5460
CAGGTCGCAT CTGCGCTTAC TGATTTGAAG AAGGAAGGGC TGGAAATGAA GAGCTAA       5517
```

This DNA molecule is known as the dspE gene for *Erwinia amylovora*. This isolated DNA molecule of the present invention encodes a protein or polypeptide which elicits a plant pathogen's hypersensitive response having an amino acid sequence of SEQ. ID. No. 8 as follows:

```
Met Glu Leu Lys Ser Leu Gly Thr Glu His Lys Ala Ala Val His Thr
1               5                   10                  15

Ala Ala His Asn Pro Val Gly His Gly Val Ala Leu Gln Gln Gly Ser
            20                  25                  30

Ser Ser Ser Ser Pro Gln Asn Ala Ala Ala Ser Leu Ala Ala Glu Gly
        35                  40                  45

Lys Asn Arg Gly Lys Met Pro Arg Ile His Gln Pro Ser Thr Ala Ala
    50                  55                  60

Asp Gly Ile Ser Ala Ala His Gln Gln Lys Lys Ser Phe Ser Leu Arg
65                  70                  75                  80

Gly Cys Leu Gly Thr Lys Lys Phe Ser Arg Ser Ala Pro Gln Gly Gln
                85                  90                  95

Pro Gly Thr Thr His Ser Lys Gly Ala Thr Leu Arg Asp Leu Leu Ala
            100                 105                 110

Arg Asp Asp Gly Glu Thr Gln His Glu Ala Ala Ala Pro Asp Ala Ala
        115                 120                 125

Arg Leu Thr Arg Ser Gly Gly Val Lys Arg Arg Asn Met Asp Asp Met
    130                 135                 140
```

```
Ala Gly Arg Pro Met Val Lys Gly Gly Ser Gly Glu Asp Lys Val Pro
145                 150                 155                 160

Thr Gln Gln Lys Arg His Gln Leu Asn Asn Phe Gly Gln Met Arg Gln
                165                 170                 175

Thr Met Leu Ser Lys Met Ala His Pro Ala Ser Ala Asn Ala Gly Asp
            180                 185                 190

Arg Leu Gln His Ser Pro Pro His Ile Pro Gly Ser His His Glu Ile
        195                 200                 205

Lys Glu Glu Pro Val Gly Ser Thr Ser Lys Ala Thr Thr Ala His Ala
    210                 215                 220

Asp Arg Val Glu Ile Ala Gln Glu Asp Asp Asp Ser Glu Phe Gln Gln
225                 230                 235                 240

Leu His Gln Gln Arg Leu Ala Arg Glu Arg Glu Asn Pro Pro Gln Pro
                245                 250                 255

Pro Lys Leu Gly Val Ala Thr Pro Ile Ser Ala Arg Phe Gln Pro Lys
            260                 265                 270

Leu Thr Ala Val Ala Glu Ser Val Leu Glu Gly Thr Asp Thr Thr Gln
        275                 280                 285

Ser Pro Leu Lys Pro Gln Ser Met Leu Lys Gly Ser Gly Ala Gly Val
    290                 295                 300

Thr Pro Leu Ala Val Thr Leu Asp Lys Gly Lys Leu Gln Leu Ala Pro
305                 310                 315                 320

Asp Asn Pro Pro Ala Leu Asn Thr Leu Leu Lys Gln Thr Leu Gly Lys
                325                 330                 335

Asp Thr Gln His Tyr Leu Ala His His Ala Ser Ser Asp Gly Ser Gln
            340                 345                 350

His Leu Leu Leu Asp Asn Lys Gly His Leu Phe Asp Ile Lys Ser Thr
        355                 360                 365

Ala Thr Ser Tyr Ser Val Leu His Asn Ser His Pro Gly Glu Ile Lys
    370                 375                 380

Gly Lys Leu Ala Gln Ala Gly Thr Gly Ser Val Ser Val Asp Gly Lys
385                 390                 395                 400

Ser Gly Lys Ile Ser Leu Gly Ser Gly Thr Gln Ser His Asn Lys Thr
                405                 410                 415

Met Leu Ser Gln Pro Gly Glu Ala His Arg Ser Leu Leu Thr Gly Ile
            420                 425                 430

Trp Gln His Pro Ala Gly Ala Ala Arg Pro Gln Gly Glu Ser Ile Arg
        435                 440                 445

Leu His Asp Asp Lys Ile His Ile Leu His Pro Glu Leu Gly Val Trp
    450                 455                 460

Gln Ser Ala Asp Lys Asp Thr His Ser Gln Leu Ser Arg Gln Ala Asp
465                 470                 475                 480

Gly Lys Leu Tyr Ala Leu Lys Asp Asn Arg Thr Leu Gln Asn Leu Ser
                485                 490                 495
```

```
Asp Asn Lys Ser Ser Glu Lys Leu Val Asp Lys Ile Lys Ser Tyr Ser
            500                 505                 510

Val Asp Gln Arg Gly Gln Val Ala Ile Leu Thr Asp Thr Pro Gly Arg
        515                 520                 525

His Lys Met Ser Ile Met Pro Ser Leu Asp Ala Ser Pro Glu Ser His
    530                 535                 540

Ile Ser Leu Ser Leu His Phe Ala Asp Ala His Gln Gly Leu Leu His
545                 550                 555                 560

Gly Lys Ser Glu Leu Glu Ala Gln Ser Val Ala Ile Ser His Gly Arg
                565                 570                 575

Leu Val Val Ala Asp Ser Glu Gly Lys Leu Phe Ser Ala Ala Ile Pro
            580                 585                 590

Lys Gln Gly Asp Gly Asn Glu Leu Lys Met Lys Ala Met Pro Gln His
        595                 600                 605

Ala Leu Asp Glu His Phe Gly His Asp His Gln Ile Ser Gly Phe Phe
    610                 615                 620

His Asp Asp His Gly Gln Leu Asn Ala Leu Val Lys Asn Asn Phe Arg
625                 630                 635                 640

Gln Gln His Ala Cys Pro Leu Gly Asn Asp His Gln Phe His Pro Gly
                645                 650                 655

Trp Asn Leu Thr Asp Ala Leu Val Ile Asp Asn Gln Leu Gly Leu His
            660                 665                 670

His Thr Asn Pro Glu Pro His Glu Ile Leu Asp Met Gly His Leu Gly
        675                 680                 685

Ser Leu Ala Leu Gln Glu Gly Lys Leu His Tyr Phe Asp Gln Leu Thr
    690                 695                 700

Lys Gly Trp Thr Gly Ala Glu Ser Asp Cys Lys Gln Leu Lys Lys Gly
705                 710                 715                 720

Leu Asp Gly Ala Ala Tyr Leu Leu Lys Asp Gly Glu Val Lys Arg Leu
                725                 730                 735

Asn Ile Asn Gln Ser Thr Ser Ser Ile Lys His Gly Thr Glu Asn Val
            740                 745                 750

Phe Ser Leu Pro His Val Arg Asn Lys Pro Glu Pro Gly Asp Ala Leu
        755                 760                 765

Gln Gly Leu Asn Lys Asp Asp Lys Ala Gln Ala Met Ala Val Ile Gly
    770                 775                 780

Val Asn Lys Tyr Leu Ala Leu Thr Glu Lys Gly Asp Ile Arg Ser Phe
785                 790                 795                 800

Gln Ile Lys Pro Gly Thr Gln Gln Leu Glu Arg Pro Ala Gln Thr Leu
                805                 810                 815

Ser Arg Glu Gly Ile Ser Gly Glu Leu Lys Asp Ile His Val Asp His
            820                 825                 830

Lys Gln Asn Leu Tyr Ala Leu Thr His Glu Gly Glu Val Phe His Gln
        835                 840                 845
```

```
Pro Arg Glu Ala Trp Gln Asn Gly Ala Glu Ser Ser Ser Trp His Lys
    850                 855                 860

Leu Ala Leu Pro Gln Ser Glu Ser Lys Leu Lys Ser Leu Asp Met Ser
865                 870                 875                 880

His Glu His Lys Pro Ile Ala Thr Phe Glu Asp Gly Ser Gln His Gln
                885                 890                 895

Leu Lys Ala Gly Gly Trp His Ala Tyr Ala Ala Pro Glu Arg Gly Pro
            900                 905                 910

Leu Ala Val Gly Thr Ser Gly Ser Gln Thr Val Phe Asn Arg Leu Met
        915                 920                 925

Gln Gly Val Lys Gly Lys Val Ile Pro Gly Ser Gly Leu Thr Val Lys
    930                 935                 940

Leu Ser Ala Gln Thr Gly Gly Met Thr Gly Ala Glu Gly Arg Lys Val
945                 950                 955                 960

Ser Ser Lys Phe Ser Glu Arg Ile Arg Ala Tyr Ala Phe Asn Pro Thr
                965                 970                 975

Met Ser Thr Pro Arg Pro Ile Lys Asn Ala Ala Tyr Ala Thr Gln His
            980                 985                 990

Gly Trp Gln Gly Arg Glu Gly Leu Lys Pro Leu Tyr Glu Met Gln Gly
        995                 1000                1005

Ala Leu Ile Lys Gln Leu Asp Ala His Asn Val Arg His Asn Ala Pro
    1010                1015                1020

Gln Pro Asp Leu Gln Ser Lys Leu Glu Thr Leu Asp Leu Gly Glu His
1025                1030                1035                1040

Gly Ala Glu Leu Leu Asn Asp Met Lys Arg Phe Arg Asp Glu Leu Glu
                1045                1050                1055

Gln Ser Ala Thr Arg Ser Val Thr Val Leu Gly Gln His Gln Gly Val
            1060                1065                1070

Leu Lys Ser Asn Gly Glu Ile Asn Ser Glu Phe Lys Pro Ser Pro Gly
        1075                1080                1085

Lys Ala Leu Val Gln Ser Phe Asn Val Asn Arg Ser Gly Gln Asp Leu
    1090                1095                1100

Ser Lys Ser Leu Gln Gln Ala Val His Ala Thr Pro Pro Ser Ala Glu
1105                1110                1115                1120

Ser Lys Leu Gln Ser Met Leu Gly His Phe Val Ser Ala Gly Val Asp
                1125                1130                1135

Met Ser His Gln Lys Gly Glu Ile Pro Leu Gly Arg Gln Arg Asp Pro
            1140                1145                1150

Asn Asp Lys Thr Ala Leu Thr Lys Ser Arg Leu Ile Leu Asp Thr Val
        1155                1160                1165

Thr Ile Gly Glu Leu His Glu Leu Ala Asp Lys Ala Lys Leu Val Ser
    1170                1175                1180

Asp His Lys Pro Asp Ala Asp Gln Ile Lys Gln Leu Arg Gln Gln Phe
1185                1190                1195                1200
```

Asp Thr Leu Arg Glu Lys Arg Tyr Glu Ser Asn Pro Val Lys His Tyr
1205 1210 1215

Thr Asp Met Gly Phe Thr His Asn Lys Ala Leu Glu Ala Asn Tyr Asp
1220 1225 1230

Ala Val Lys Ala Phe Ile Asn Ala Phe Lys Lys Glu His His Gly Val
1235 1240 1245

Asn Leu Thr Thr Arg Thr Val Leu Glu Ser Gln Gly Ser Ala Glu Leu
1250 1255 1260

Ala Lys Lys Leu Lys Asn Thr Leu Leu Ser Leu Asp Ser Gly Glu Ser
1265 1270 1275 1280

Met Ser Phe Ser Arg Ser Tyr Gly Gly Gly Val Ser Thr Val Phe Val
1285 1290 1295

Pro Thr Leu Ser Lys Lys Val Pro Val Pro Val Ile Pro Gly Ala Gly
1300 1305 1310

Ile Thr Leu Asp Arg Ala Tyr Asn Leu Ser Phe Ser Arg Thr Ser Gly
1315 1320 1325

Gly Leu Asn Val Ser Phe Gly Arg Asp Gly Gly Val Ser Gly Asn Ile
1330 1335 1340

Met Val Ala Thr Gly His Asp Val Met Pro Tyr Met Thr Gly Lys Lys
1345 1350 1355 1360

Thr Ser Ala Gly Asn Ala Ser Asp Trp Leu Ser Ala Lys His Lys Ile
1365 1370 1375

Ser Pro Asp Leu Arg Ile Gly Ala Ala Val Ser Gly Thr Leu Gln Gly
1380 1385 1390

Thr Leu Gln Asn Ser Leu Lys Phe Lys Leu Thr Glu Asp Glu Leu Pro
1395 1400 1405

Gly Phe Ile His Gly Leu Thr His Gly Thr Leu Thr Pro Ala Glu Leu
1410 1415 1420

Leu Gln Lys Gly Ile Glu His Gln Met Lys Gln Gly Ser Lys Leu Thr
1425 1430 1435 1440

Phe Ser Val Asp Thr Ser Ala Asn Leu Asp Leu Arg Ala Gly Ile Asn
1445 1450 1455

Leu Asn Glu Asp Gly Ser Lys Pro Asn Gly Val Thr Ala Arg Val Ser
1460 1465 1470

Ala Gly Leu Ser Ala Ser Ala Asn Leu Ala Ala Gly Ser Arg Glu Arg
1475 1480 1485

Ser Thr Thr Ser Gly Gln Phe Gly Ser Thr Thr Ser Ala Ser Asn Asn
1490 1495 1500

Arg Pro Thr Phe Leu Asn Gly Val Gly Ala Gly Ala Asn Leu Thr Ala
1505 1510 1515 1520

Ala Leu Gly Val Ala His Ser Ser Thr His Glu Gly Lys Pro Val Gly
1525 1530 1535

```
Ile Phe Pro Ala Phe Thr Ser Thr Asn Val Ser Ala Ala Leu Ala Leu
        1540                1545                1550

Asp Asn Arg Thr Ser Gln Ser Ile Ser Leu Glu Leu Lys Arg Ala Glu
    1555                1560                1565

Pro Val Thr Ser Asn Asp Ile Ser Glu Leu Thr Ser Thr Leu Gly Lys
    1570                1575                1580

His Phe Lys Asp Ser Ala Thr Thr Lys Met Leu Ala Ala Leu Lys Glu
1585                1590                1595                1600

Leu Asp Asp Ala Lys Pro Ala Glu Gln Leu His Ile Leu Gln Gln His
                1605                1610                1615

Phe Ser Ala Lys Asp Val Val Gly Asp Glu Arg Tyr Glu Ala Val Arg
            1620                1625                1630

Asn Leu Lys Lys Leu Val Ile Arg Gln Gln Ala Ala Asp Ser His Ser
        1635                1640                1645

Met Glu Leu Gly Ser Ala Ser His Ser Thr Thr Tyr Asn Asn Leu Ser
    1650                1655                1660

Arg Ile Asn Asn Asp Gly Ile Val Glu Leu Leu His Lys His Phe Asp
1665                1670                1675                1680

Ala Ala Leu Pro Ala Ser Ser Ala Lys Arg Leu Gly Glu Met Met Asn
                1685                1690                1695

Asn Asp Pro Ala Leu Lys Asp Ile Ile Lys Gln Leu Gln Ser Thr Pro
            1700                1705                1710

Phe Ser Ser Ala Ser Val Ser Met Glu Leu Lys Asp Gly Leu Arg Glu
        1715                1720                1725

Gln Thr Glu Lys Ala Ile Leu Asp Gly Lys Val Gly Arg Glu Glu Val
    1730                1735                1740

Gly Val Leu Phe Gln Asp Arg Asn Asn Leu Arg Val Lys Ser Val Ser
1745                1750                1755                1760

Val Ser Gln Ser Val Ser Lys Ser Glu Gly Phe Asn Thr Pro Ala Leu
                1765                1770                1775

Leu Leu Gly Thr Ser Asn Ser Ala Ala Met Ser Met Glu Arg Asn Ile
            1780                1785                1790

Gly Thr Ile Asn Phe Lys Tyr Gly Gln Asp Gln Asn Thr Pro Arg Arg
        1795                1800                1805

Phe Thr Leu Glu Gly Gly Ile Ala Gln Ala Asn Pro Gln Val Ala Ser
    1810                1815                1820

Ala Leu Thr Asp Leu Lys Lys Glu Gly Leu Glu Met Lys Ser
1825                1830                1835
```

This protein or polypeptide is about 198 kDa and has a pI of 8.98.

The present invention relates to an isolated DNA molecule having a nucleotide sequence of SEQ. ID. No. 9 as follows:

```
ATGACATCGT CACAGCAGCG GGTTGAAAGG TTTTTACAGT ATTTCTCCGC CGGGTGTAAA     60
ACGCCCATAC ATCTGAAAGA CGGGGTGTGC GCCCTGTATA ACGAACAAGA TGAGGAGGCG    120
GCGGTGCTGG AAGTACCGCA ACACAGCGAC AGCCTGTTAC TACACTGCCG AATCATTGAG    180
GCTGACCCAC AAACTTCAAT AACCCTGTAT TCGATGCTAT TACAGCTGAA TTTTGAAATG    240
GCGGCCATGC GCGGCTGTTG GCTGGCGCTG GATGAACTGC ACAACGTGCG TTTATGTTTT    300
CAGCAGTCGC TGGAGCATCT GGATGAAGCA AGTTTTAGCG ATATCGTTAG CGGCTTCATC    360
GAACATGCGG CAGAAGTGCG TGAGTATATA GCGCAATTAG ACGAGAGTAG CGCGGCATAA    420
```

This is known as the dspF gene. This isolated DNA molecule of the present invention encodes a hypersensitive response elicitor protein or polypeptide having an amino acid sequence of SEQ. ID. No. 10 as follows:

```
Met Thr Ser Ser Gln Gln Arg Val Glu Arg Phe Leu Gln Tyr Phe Ser
1               5                   10                  15

Ala Gly Cys Lys Thr Pro Ile His Leu Lys Asp Gly Val Cys Ala Leu
            20                  25                  30

Tyr Asn Glu Gln Asp Glu Glu Ala Ala Val Leu Glu Val Pro Gln His
        35                  40                  45

Ser Asp Ser Leu Leu Leu His Cys Arg Ile Ile Glu Ala Asp Pro Gln
    50                  55                  60

Thr Ser Ile Thr Leu Tyr Ser Met Leu Leu Gln Leu Asn Phe Glu Met
65                  70                  75                  80

Ala Ala Met Arg Gly Cys Trp Leu Ala Leu Asp Glu Leu His Asn Val
                85                  90                  95

Arg Leu Cys Phe Gln Gln Ser Leu Glu His Leu Asp Glu Ala Ser Phe
            100                 105                 110

Ser Asp Ile Val Ser Gly Phe Ile Glu His Ala Ala Glu Val Arg Glu
        115                 120                 125

Tyr Ile Ala Gln Leu Asp Glu Ser Ser Ala Ala
    130                 135
```

This protein or polypeptide is about 16 kDa and has a pI of 4.45.

The hypersensitive response elicitor polypeptide or protein derived from *Pseudomonas syringae* has an amino acid sequence corresponding to SEQ. ID. No. 11 as follows:

```
Met Gln Ser Leu Ser Leu Asn Ser Ser Ser Leu Gln Thr Pro Ala Met
1               5                   10                  15
```

```
Ala Leu Val Leu Val Arg Pro Glu Ala Glu Thr Thr Gly Ser Thr Ser
            20                  25                  30

Ser Lys Ala Leu Gln Glu Val Val Val Lys Leu Ala Glu Glu Leu Met
        35                  40                  45

Arg Asn Gly Gln Leu Asp Asp Ser Ser Pro Leu Gly Lys Leu Leu Ala
    50                  55                  60

Lys Ser Met Ala Ala Asp Gly Lys Ala Gly Gly Gly Ile Glu Asp Val
65                  70                  75                  80

Ile Ala Ala Leu Asp Lys Leu Ile His Glu Lys Leu Gly Asp Asn Phe
                85                  90                  95

Gly Ala Ser Ala Asp Ser Ala Ser Gly Thr Gly Gln Gln Asp Leu Met
            100                 105                 110

Thr Gln Val Leu Asn Gly Leu Ala Lys Ser Met Leu Asp Asp Leu Leu
        115                 120                 125

Thr Lys Gln Asp Gly Gly Thr Ser Phe Ser Glu Asp Asp Met Pro Met
    130                 135                 140

Leu Asn Lys Ile Ala Gln Phe Met Asp Asp Asn Pro Ala Gln Phe Pro
145                 150                 155                 160

Lys Pro Asp Ser Gly Ser Trp Val Asn Glu Leu Lys Glu Asp Asn Phe
                165                 170                 175

Leu Asp Gly Asp Glu Thr Ala Ala Phe Arg Ser Ala Leu Asp Ile Ile
            180                 185                 190

Gly Gln Gln Leu Gly Asn Gln Gln Ser Asp Ala Gly Ser Leu Ala Gly
        195                 200                 205

Thr Gly Gly Gly Leu Gly Thr Pro Ser Ser Phe Ser Asn Asn Ser Ser
    210                 215                 220

Val Met Gly Asp Pro Leu Ile Asp Ala Asn Thr Gly Pro Gly Asp Ser
225                 230                 235                 240

Gly Asn Thr Arg Gly Glu Ala Gly Gln Leu Ile Gly Glu Leu Ile Asp
                245                 250                 255

Arg Gly Leu Gln Ser Val Leu Ala Gly Gly Gly Leu Gly Thr Pro Val
            260                 265                 270

Asn Thr Pro Gln Thr Gly Thr Ser Ala Asn Gly Gly Gln Ser Ala Gln
        275                 280                 285

Asp Leu Asp Gln Leu Leu Gly Gly Leu Leu Leu Lys Gly Leu Glu Ala
    290                 295                 300

Thr Leu Lys Asp Ala Gly Gln Thr Gly Thr Asp Val Gln Ser Ser Ala
305                 310                 315                 320
```

```
Ala Gln Ile Ala Thr Leu Leu Val Ser Thr Leu Leu Gln Gly Thr Arg
                325                 330                 335

Asn Gln Ala Ala Ala
            340
```

This hypersensitive response elicitor polypeptide or protein has a molecular weight of 34-35 kDa. It is rich in glycine (about 13.5%) and lacks cysteine and tyrosine. Further information about the hypersensitive response elicitor derived from *Pseudomonas syringae* is found in He, S. Y., H. C. Huang, and A. Collmer, "*Pseudomonas syringae* pv. *syringae* Harpin$_{Pss}$: a Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," Cell 73:1255-1266 (1993), which is hereby incorporated by reference. The DNA molecule encoding the hypersensitive response elicitor from *Pseudomonas syringae* has a nucleotide sequence corresponding to SEQ. ID. No. 12 as follows:

```
ATGCAGAGTC TCAGTCTTAA CAGCAGCTCG CTGCAAACCC CGGCAATGGC CCTTGTCCTG     60
GTACGTCCTG AAGCCGAGAC GACTGGCAGT ACGTCGAGCA AGGCGCTTCA GGAAGTTGTC    120
GTGAAGCTGG CCGAGGAACT GATGCGCAAT GGTCAACTCG ACGACAGCTC GCCATTGGGA    180
AAACTGTTGG CCAAGTCGAT GGCCGCAGAT GGCAAGGCGG GCGGCGGTAT TGAGGATGTC    240
ATCGCTGCGC TGGACAAGCT GATCCATGAA AAGCTCGGTG ACAACTTCGG CGCGTCTGCG    300
GACAGCGCCT CGGGTACCGG ACAGCAGGAC CTGATGACTC AGGTGCTCAA TGGCCTGGCC    360
AAGTCGATGC TCGATGATCT TCTGACCAAG CAGGATGGCG GGACAAGCTT CTCCGAAGAC    420
GATATGCCGA TGCTGAACAA GATCGCGCAG TTCATGGATG ACAATCCCGC ACAGTTTCCC    480
AAGCCGGACT CGGGCTCCTG GGTGAACGAA CTCAAGGAAG ACAACTTCCT TGATGGCGAC    540
GAAACGGCTG CGTTCCGTTC GGCACTCGAC ATCATTGGCC AGCAACTGGG TAATCAGCAG    600
AGTGACGCTG GCAGTCTGGC AGGGACGGGT GGAGGTCTGG GCACTCCGAG CAGTTTTTCC    660
AACAACTCGT CCGTGATGGG TGATCCGCTG ATCGACGCCA ATACCGGTCC CGGTGACAGC    720
GGCAATACCC GTGGTGAAGC GGGGCAACTG ATCGGCGAGC TTATCGACCG TGGCCTGCAA    780
TCGGTATTGG CCGGTGGTGG ACTGGGCACA CCCGTAAACA CCCCGCAGAC CGGTACGTCG    840
GCGAATGGCG GACAGTCCGC TCAGGATCTT GATCAGTTGC TGGGCGGCTT GCTGCTCAAG    900
GGCCTGGAGG CAACGCTCAA GGATGCCGGG CAAACAGGCA CCGACGTGCA GTCGAGCGCT    960
GCGCAAATCG CCACCTTGCT GGTCAGTACG CTGCTGCAAG GCACCCGCAA TCAGGCTGCA   1020
GCCTGA                                                               1026
```

Another potentially suitable hypersensitive response elicitor from *Pseudomonas syringae* is disclosed in U.S. Patent Application Serial No. 09/120,817, which is hereby incorporated by reference. The protein has a nucleotide sequence of SEQ. ID. No. 13 as follows:

```
TCCACTTCGC TGATTTTGAA ATTGGCAGAT TCATAGAAAC GTTCAGGTGT GGAAATCAGG     60
CTGAGTGCGC AGATTTCGTT GATAAGGGTG TGGTACTGGT CATTGTTGGT CATTTCAAGG    120
CCTCTGAGTG CGGTGCGGAG CAATACCAGT CTTCCTGCTG GCGTGTGCAC ACTGAGTCGC    180
AGGCATAGGC ATTTCAGTTC CTTGCGTTGG TTGGGCATAT AAAAAAAGGA ACTTTTAAAA    240
ACAGTGCAAT GAGATGCCGG CAAAACGGGA ACCGGTCGCT GCGCTTTGCC ACTCACTTCG    300
AGCAAGCTCA ACCCCAAACA TCCACATCCC TATCGAACGG ACAGCGATAC GGCCACTTGC    360
TCTGGTAAAC CCTGGAGCTG GCGTCGGTCC AATTGCCCAC TTAGCGAGGT AACGCAGCAT    420
GAGCATCGGC ATCACACCCC GGCCGCAACA GACCACCACG CCACTCGATT TTTCGGCGCT    480
AAGCGGCAAG AGTCCTCAAC CAAACACGTT CGGCGAGCAG AACACTCAGC AAGCGATCGA    540
CCCGAGTGCA CTGTTGTTCG GCAGCGACAC ACAGAAAGAC GTCAACTTCG GCACGCCCGA    600
CAGCACCGTC CAGAATCCGC AGGACGCCAG CAAGCCCAAC GACAGCCAGT CCAACATCGC    660
TAAATTGATC AGTGCATTGA TCATGTCGTT GCTGCAGATG CTCACCAACT CCAATAAAAA    720
GCAGGACACC AATCAGGAAC AGCCTGATAG CCAGGCTCCT TTCCAGAACA ACGGCGGGCT    780
CGGTACACCG TCGGCCGATA GCGGGGGCGG CGGTACACCG GATGCGACAG GTGGCGGCGG    840
CGGTGATACG CCAAGCGCAA CAGGCGGTGG CGGCGGTGAT ACTCCGACCG CAACAGGCGG    900
TGGCGGCAGC GGTGGCGGCG GCACACCCAC TGCAACAGGT GGCGGCAGCG GTGGCACACC    960
CACTGCAACA GGCGGTGGCG AGGGTGGCGT AACACCGCAA ATCACTCCGC AGTTGGCCAA   1020
CCCTAACCGT ACCTCAGGTA CTGGCTCGGT GTCGGACACC GCAGGTTCTA CCGAGCAAGC   1080
CGGCAAGATC AATGTGGTGA AAGACACCAT CAAGGTCGGC GCTGGCGAAG TCTTTGACGG   1140
CCACGGCGCA ACCTTCACTG CCGACAAATC TATGGGTAAC GGAGACCAGG GCGAAAATCA   1200
GAAGCCCATG TTCGAGCTGG CTGAAGGCGC TACGTTGAAG AATGTGAACC TGGGTGAGAA   1260
CGAGGTCGAT GGCATCCACG TGAAAGCCAA AAACGCTCAG GAAGTCACCA TTGACAACGT   1320
GCATGCCCAG AACGTCGGTG AAGACCTGAT TACGGTCAAA GGCGAGGGAG GCGCAGCGGT   1380
CACTAATCTG AACATCAAGA ACAGCAGTGC CAAAGGTGCA GACGACAAGG TTGTCCAGCT   1440
CAACGCCAAC ACTCACTTGA AAATCGACAA CTTCAAGGCC GACGATTTCG GCACGATGGT   1500
TCGCACCAAC GGTGGCAAGC AGTTTGATGA CATGAGCATC GAGCTGAACG GCATCGAAGC   1560
TAACCACGGC AAGTTCGCCC TGGTGAAAAG CGACAGTGAC GATCTGAAGC TGGCAACGGG   1620
```

```
CAACATCGCC ATGACCGACG TCAAACACGC CTACGATAAA ACCCAGGCAT CGACCCAACA     1680

CACCGAGCTT TGAATCCAGA CAAGTAGCTT GAAAAAAGGG GGTGGACTC                 1729
```

This DNA molecule is known as the dspE gene for *Pseudomonas syringae*. This isolated DNA molecule of the present invention encodes a protein or polypeptide which elicits a plant pathogen's hypersensitive response having an amino acid sequence of SEQ. ID. No. 14 as follows:

```
Met Ser Ile Gly Ile Thr Pro Arg Pro Gln Gln Thr Thr Thr Pro Leu
1               5                   10                  15

Asp Phe Ser Ala Leu Ser Gly Lys Ser Pro Gln Pro Asn Thr Phe Gly
            20                  25                  30

Glu Gln Asn Thr Gln Gln Ala Ile Asp Pro Ser Ala Leu Leu Phe Gly
        35                  40                  45

Ser Asp Thr Gln Lys Asp Val Asn Phe Gly Thr Pro Asp Ser Thr Val
    50                  55                  60

Gln Asn Pro Gln Asp Ala Ser Lys Pro Asn Asp Ser Gln Ser Asn Ile
65                  70                  75                  80

Ala Lys Leu Ile Ser Ala Leu Ile Met Ser Leu Leu Gln Met Leu Thr
                85                  90                  95

Asn Ser Asn Lys Lys Gln Asp Thr Asn Gln Glu Gln Pro Asp Ser Gln
            100                 105                 110

Ala Pro Phe Gln Asn Asn Gly Gly Leu Gly Thr Pro Ser Ala Asp Ser
        115                 120                 125

Gly Gly Gly Gly Thr Pro Asp Ala Thr Gly Gly Gly Gly Gly Asp Thr
    130                 135                 140

Pro Ser Ala Thr Gly Gly Gly Gly Gly Asp Thr Pro Thr Ala Thr Gly
145                 150                 155                 160

Gly Gly Gly Ser Gly Gly Gly Gly Thr Pro Thr Ala Thr Gly Gly Gly
                165                 170                 175

Ser Gly Gly Thr Pro Thr Ala Thr Gly Gly Gly Glu Gly Gly Val Thr
            180                 185                 190

Pro Gln Ile Thr Pro Gln Leu Ala Asn Pro Asn Arg Thr Ser Gly Thr
        195                 200                 205

Gly Ser Val Ser Asp Thr Ala Gly Ser Thr Glu Gln Ala Gly Lys Ile
    210                 215                 220

Asn Val Val Lys Asp Thr Ile Lys Val Gly Ala Gly Glu Val Phe Asp
225                 230                 235                 240
```

```
Gly His Gly Ala Thr Phe Thr Ala Asp Lys Ser Met Gly Asn Gly Asp
                245                 250                 255

Gln Gly Glu Asn Gln Lys Pro Met Phe Glu Leu Ala Glu Gly Ala Thr
            260                 265                 270

Leu Lys Asn Val Asn Leu Gly Glu Asn Glu Val Asp Gly Ile His Val
        275                 280                 285

Lys Ala Lys Asn Ala Gln Glu Val Thr Ile Asp Asn Val His Ala Gln
    290                 295                 300

Asn Val Gly Glu Asp Leu Ile Thr Val Lys Gly Glu Gly Gly Ala Ala
305                 310                 315                 320

Val Thr Asn Leu Asn Ile Lys Asn Ser Ser Ala Lys Gly Ala Asp Asp
                325                 330                 335

Lys Val Val Gln Leu Asn Ala Asn Thr His Leu Lys Ile Asp Asn Phe
            340                 345                 350

Lys Ala Asp Asp Phe Gly Thr Met Val Arg Thr Asn Gly Gly Lys Gln
        355                 360                 365

Phe Asp Asp Met Ser Ile Glu Leu Asn Gly Ile Glu Ala Asn His Gly
    370                 375                 380

Lys Phe Ala Leu Val Lys Ser Asp Ser Asp Asp Leu Lys Leu Ala Thr
385                 390                 395                 400

Gly Asn Ile Ala Met Thr Asp Val Lys His Ala Tyr Asp Lys Thr Gln
                405                 410                 415

Ala Ser Thr Gln His Thr Glu Leu
            420
```

This protein or polypeptide is about 42.9 kDa.

The hypersensitive response elicitor polypeptide or protein derived from *Pseudomonas solanacearum* has an amino acid sequence corresponding to SEQ. ID. No. 15 as follows:

```
Met Ser Val Gly Asn Ile Gln Ser Pro Ser Asn Leu Pro Gly Leu Gln
1               5                   10                  15

Asn Leu Asn Leu Asn Thr Asn Thr Asn Ser Gln Gln Ser Gly Gln Ser
            20                  25                  30

Val Gln Asp Leu Ile Lys Gln Val Glu Lys Asp Ile Leu Asn Ile Ile
        35                  40                  45

Ala Ala Leu Val Gln Lys Ala Ala Gln Ser Ala Gly Gly Asn Thr Gly
    50                  55                  60
```

```
Asn Thr Gly Asn Ala Pro Ala Lys Asp Gly Asn Ala Asn Ala Gly Ala
65                  70                  75                  80

Asn Asp Pro Ser Lys Asn Asp Pro Ser Lys Ser Gln Ala Pro Gln Ser
                85                  90                  95

Ala Asn Lys Thr Gly Asn Val Asp Asp Ala Asn Asn Gln Asp Pro Met
            100                 105                 110

Gln Ala Leu Met Gln Leu Leu Glu Asp Leu Val Lys Leu Leu Lys Ala
        115                 120                 125

Ala Leu His Met Gln Gln Pro Gly Gly Asn Asp Lys Gly Asn Gly Val
    130                 135                 140

Gly Gly Ala Asn Gly Ala Lys Gly Ala Gly Gly Gln Gly Gly Leu Ala
145                 150                 155                 160

Glu Ala Leu Gln Glu Ile Glu Gln Ile Leu Ala Gln Leu Gly Gly Gly
                165                 170                 175

Gly Ala Gly Ala Gly Gly Ala Gly Gly Gly Val Gly Gly Ala Gly Gly
            180                 185                 190

Ala Asp Gly Gly Ser Gly Ala Gly Gly Ala Gly Gly Ala Asn Gly Ala
        195                 200                 205

Asp Gly Gly Asn Gly Val Asn Gly Asn Gln Ala Asn Gly Pro Gln Asn
    210                 215                 220

Ala Gly Asp Val Asn Gly Ala Asn Gly Ala Asp Asp Gly Ser Glu Asp
225                 230                 235                 240

Gln Gly Gly Leu Thr Gly Val Leu Gln Lys Leu Met Lys Ile Leu Asn
                245                 250                 255

Ala Leu Val Gln Met Met Gln Gln Gly Gly Leu Gly Gly Gly Asn Gln
            260                 265                 270

Ala Gln Gly Gly Ser Lys Gly Ala Gly Asn Ala Ser Pro Ala Ser Gly
        275                 280                 285

Ala Asn Pro Gly Ala Asn Gln Pro Gly Ser Ala Asp Asp Gln Ser Ser
    290                 295                 300

Gly Gln Asn Asn Leu Gln Ser Gln Ile Met Asp Val Val Lys Glu Val
305                 310                 315                 320

Val Gln Ile Leu Gln Gln Met Leu Ala Ala Gln Asn Gly Gly Ser Gln
                325                 330                 335

Gln Ser Thr Ser Thr Gln Pro Met
            340
```

It is encoded by a DNA molecule having a nucleotide sequence corresponding SEQ. ID. No. 16 as follows:

```
ATGTCAGTCG GAAACATCCA GAGCCCGTCG AACCTCCCGG GTCTGCAGAA CCTGAACCTC     60
AACACCAACA CCAACAGCCA GCAATCGGGC CAGTCCGTGC AAGACCTGAT CAAGCAGGTC    120
GAGAAGGACA TCCTCAACAT CATCGCAGCC CTCGTGCAGA AGGCCGCACA GTCGGCGGGC    180
GGCAACACCG GTAACACCGG CAACGCGCCG GCGAAGGACG GCAATGCCAA CGCGGGCGCC    240
AACGACCCGA GCAAGAACGA CCCGAGCAAG AGCCAGGCTC CGCAGTCGGC CAACAAGACC    300
GGCAACGTCG ACGACGCCAA CAACCAGGAT CCGATGCAAG CGCTGATGCA GCTGCTGGAA    360
GACCTGGTGA AGCTGCTGAA GGCGGCCCTG CACATGCAGC AGCCCGGCGG CAATGACAAG    420
GGCAACGGCG TGGGCGGTGC CAACGGCGCC AAGGGTGCCG GCGGCCAGGG CGGCCTGGCC    480
GAAGCGCTGC AGGAGATCGA GCAGATCCTC GCCCAGCTCG GCGGCGGCGG TGCTGGCGCC    540
GGCGGCGCGG GTGGCGGTGT CGGCGGTGCT GGTGGCGCGG ATGGCGGCTC CGGTGCGGGT    600
GGCGCAGGCG GTGCGAACGG CGCCGACGGC GGCAATGGCG TGAACGGCAA CCAGGCGAAC    660
GGCCCGCAGA ACGCAGGCGA TGTCAACGGT GCCAACGGCG CGGATGACGG CAGCGAAGAC    720
CAGGGCGGCC TCACCGGCGT GCTGCAAAAG CTGATGAAGA TCCTGAACGC GCTGGTGCAG    780
ATGATGCAGC AAGGCGGCCT CGGCGGCGGC AACCAGGCGC AGGGCGGCTC GAAGGGTGCC    840
GGCAACGCCT CGCCGGCTTC CGGCGCGAAC CCGGGCGCGA ACCAGCCCGG TTCGGCGGAT    900
GATCAATCGT CCGGCCAGAA CAATCTGCAA TCCCAGATCA TGGATGTGGT GAAGGAGGTC    960
GTCCAGATCC TGCAGCAGAT GCTGGCGGCG CAGAACGGCG GCAGCCAGCA GTCCACCTCG   1020
ACGCAGCCGA TGTAA                                                    1035
```

Further information regarding the hypersensitive response elicitor polypeptide or protein derived from *Pseudomonas solanacearum* is set forth in Arlat, M., F. Van Gijsegem, J. C. Huet, J. C. Pemollet, and C. A. Boucher, "PopA1, a Protein which Induces a Hypersensitive-like Response in Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-533 (1994), which is hereby incorporated by reference.

The hypersensitive response elicitor polypeptide or protein from *Xanthomonas campestris* pv. glycines has an amino acid sequence corresponding to SEQ. ID. No. 17 as follows:

```
Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Ala Ile Leu Ala
1               5                   10                  15
```

```
Ala Ile Ala Leu Pro Ala Tyr Gln Asp Tyr
                20                  25
```

This sequence is an amino terminal sequence having only 26 residues from the hypersensitive response elicitor polypeptide or protein of *Xanthomonas campestris* pv. glycines. It matches with fimbrial subunit proteins determined in other *Xanthomonas campestris* pathovars.

The hypersensitive response elicitor polypeptide or protein from *Xanthomonas campestris pv. pelargonii* is heat stable, protease sensitive, and has a molecular weight of 20 kDa. It includes an amino acid sequence corresponding to SEQ. ID. No. 18 as follows:

```
Ser Ser Gln Gln Ser Pro Ser Ala Gly Ser Glu Gln Gln Leu Asp Gln
1               5                   10                  15

Leu Leu Ala Met
            20
```

Isolation of *Erwinia carotovora* hypersensitive response elictor protein or polypeptide is described in Cui et al., "The RsmA Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrp* N_{Ecc} and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI, 9(7):565-73 (1996), which is hereby incorporated by reference. The hypersensitive response elicitor protein or polypeptide of *Erwinia stewartii* is set forth in Ahmad et al., "Harpin is Not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," 8th Int'l. Cong. Molec. Plant-Microbe Interact., July 14-19, 1996 and Ahmad, et al., "Harpin is Not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," Ann. Mtg. Am. Phytopath. Soc., July 27-31, 1996, which are hereby incorporated by reference.

Hypersensitive response elicitor proteins or polypeptides from *Phytophthora parasitica, Phytophthora cryptogea, Phytophthora cinnamoni, Phytophthora capsici, Phytophthora megasperma,* and *Phytophora citrophthora* are described in Kaman, et al., "Extracellular Protein Elicitors from Phytophthora: Most Specificity and Induction of Resistance to Bacterial and Fungal Phytopathogens," Molec. Plant-Microbe Interact., 6(1):15-25 (1993), Ricci et al., "Structure and Activity of Proteins from Pathogenic Fungi Phytophthora Eliciting Necrosis and

Acquired Resistance in Tobacco," Eur. J. Biochem., 183:555-63 (1989), Ricci et al., "Differential Production of Parasiticein, and Elicitor of Necrosis and Resistance in Tobacco, by Isolates of Phytophthora parasitica," Plant Path. 41:298-307 (1992), Baillreul et al, "A New Elicitor of the Hypersensitive Response in Tobacco: A Fungal Glycoprotein Elicits Cell Death, Expression of Defence Genes, Production of Salicylic Acid, and Induction of Systemic Acquired Resistance," Plant J., 8(4):551-60 (1995), and Bonnet et al., "Acquired Resistance Triggered by Elicitors in Tobacco and Other Plants," Eur. J. Plant Path., 102:181-92 (1996), which are hereby incorporated by reference.

Another hypersensitive response elicitor in accordance with the present invention is from *Clavibacter michiganensis* subsp. *sepedonicus* which is fully described in U.S. Patent Application Serial No. 09/136,625, which is hereby incorporated by reference.

The above elicitors are exemplary. Other elicitors can be identified by growing fungi or bacteria that elicit a hypersensitive response under conditions which genes encoding an elicitor are expressed. Cell-free preparations from culture supernatants can be tested for elicitor activity (i.e. local necrosis) by using them to infiltrate appropriate plant tissues.

Fragments of the above hypersensitive response elicitor polypeptides or proteins as well as fragments of full length elicitors from other pathogens are encompassed by the method of the present invention.

Suitable fragments can be produced by several means. In the first, subclones of the gene encoding a known elicitor protein are produced by conventional molecular genetic manipulation by subcloning gene fragments. The subclones then are expressed *in vitro* or *in vivo* in bacterial cells to yield a smaller protein or peptide that can be tested for elicitor activity according to the procedure described below.

As an alternative, fragments of an elicitor protein can be produced by digestion of a full-length elicitor protein with proteolytic enzymes like chymotrypsin or *Staphylococcus* proteinase A, or trypsin. Different proteolytic enzymes are likely to cleave elicitor proteins at different sites based on the amino acid sequence of the elicitor protein. Some of the fragments that result from proteolysis may be active elicitors of resistance.

In another approach, based on knowledge of the primary structure of the protein, fragments of the elicitor protein gene may be synthesized by using the PCR technique together with specific sets of primers chosen to represent particular portions of the protein. These then would be cloned into an appropriate vector for expression of a truncated peptide or protein.

Chemical synthesis can also be used to make suitable fragments. Such a synthesis is carried out using known amino acid sequences for the elicitor being produced. Alternatively, subjecting a full length elicitor to high temperatures and pressures will produce fragments. These fragments can then be separated by conventional procedures (e.g., chromatography, SDS-PAGE).

An example of suitable fragments of a hypersensitive response elicitor which do not elicit a hypersensitive response include fragments of the *Erwinia*. Suitable fragments include a C-terminal fragment of the amino acid sequence of SEQ. ID. No. 3, an N-terminal fragment of the amino acid sequence of SEQ. ID. No. 3, or an internal fragment of the amino acid sequence of SEQ. ID. No. 3. The C-terminal fragment of the amino acid sequence of SEQ. ID. No. 3 can span the following amino acids of SEQ. ID. No. 3: 169 and 403, 210 and 403, 267 and 403, or 343 and 403. The internal fragment of the amino acid sequence of SEQ. ID. No. 3 can span the following amino acids of SEQ. ID. No. 3: 105 and 179, 137 and 166, 121 and 150, or 137 and 156. Other suitable fragments can be identified in accordance with the present invention.

Another example of suitable fragments of a hypersensitive response elicitor which do elicit a hypersensitive response are *Erwinia amylovora* fragments including a C-terminal fragment of the amino acid sequence of SEQ. ID. No. 3, an N-terminal fragment of the amino acid sequence of SEQ. ID. No. 3, or an internal fragment of the amino acid sequence of SEQ. ID. No. 3. The C-terminal fragment of the amino acid sequence of SEQ. ID. No. 3 can span amino acids 105 and 403 of SEQ. ID. No. 3. The N-terminal fragment of the amino acid sequence of SEQ. ID. No. 3 can span the following amino acids of SEQ. ID. No. 3: 1 and 98, 1 and 104, 1 and 122, 1 and 168, 1 and 218, 1 and 266, 1 and 342, 1 and 321, and 1 and 372. The internal fragment of the amino acid sequence of SEQ. ID. No. 3 can span the

following amino acids of SEQ. ID. No. 3: 76 and 209, 105 and 209, 99 and 209, 137 and 204, 137 and 200, 109 and 204, 109 and 200, 137 and 180, and 105 and 180.

Suitable DNA molecules are those that hybridize to the DNA molecule comprising a nucleotide sequence of SEQ. ID. Nos. 2, 4, 5, 7, 9, 12, 13, and 16 under stringent conditions. An example of suitable high stringency conditions is when hybridization is carried out at 65°C for 20 hours in a medium containing 1M NaCl, 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.1% sodium dodecyl sulfate, 0.2% ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 50 μm g/ml *E. coli* DNA.

Variants may be made by, for example, the deletion or addition of amino acids that have minimal influence on the properties, secondary structure and hydropathic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the polypeptide.

The hypersensitive response elicitor of the present invention is preferably in isolated form (i.e. separated from its host organism) and more preferably produced in purified form (preferably at least about 60%, more preferably 80%, pure) by conventional techniques. Typically, the hypersensitive response elicitor of the present invention is produced but not secreted into the growth medium of recombinant host cells. Alternatively, the protein or polypeptide of the present invention is secreted into growth medium. In the case of unsecreted protein, to isolate the protein, the host cell (e.g., *E. coli*) carrying a recombinant plasmid is propagated, lysed by sonication, heat, or chemical treatment, and the homogenate is centrifuged to remove bacterial debris. The supernatant is then subjected to heat treatment and the hypersensitive response elicitor is separated by centrifugation. The supernatant fraction containing the hypersensitive response elicitor is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the fragment. If necessary, the protein fraction may be further purified by ion exchange or HPLC.

The DNA molecule encoding the hypersensitive response elicitor polypeptide or protein can be incorporated in cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule into an

expression system to which the DNA molecule is heterologous (i.e. not normally present). The heterologous DNA molecule is inserted into the expression system or vector in sense orientation and correct reading frame. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences.

U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.

Recombinant genes may also be introduced into viruses, such as vaccina virus. Recombinant viruses can be generated by transfection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC1084, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see F.W. Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," Gene Expression Technology vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1989), which is hereby incorporated by reference.

A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA;

microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

Transcription of DNA is dependent upon the presence of a promotor which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promotors differ from those of procaryotic promotors. Furthermore, eucaryotic promotors and accompanying genetic signals may not be recognized in or may not function in a procaryotic system, and, further, procaryotic promotors are not recognized and do not function in eucaryotic cells.

Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes. Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473 (1979), which is hereby incorporated by reference.

Promotors vary in their "strength" (i.e. their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promotors in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promotors may be used. For instance, when cloning in *E. coli*, its bacteriophages, or plasmids, promotors such as the T7 phage promotor, *lac* promotor,

trp promotor, *rec*A promotor, ribosomal RNA promotor, the P_R and P_L promotors of coliphage lambda and others, including but not limited, to *lac*UV5, *omp*F, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lac*UV5 (*tac*) promotor or other *E. coli* promotors produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promotor unless specifically induced. In certain operations, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the *lac* operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different controls.

Specific initiation signals are also required for efficient gene transcription and translation in procaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promotor, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires an SD sequence about 7-9 bases 5' to the initiation codon ("ATG") to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *cro* gene or the *N* gene of coliphage lambda, or from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Once the isolated DNA molecule encoding the hypersensitive response elicitor polypeptide or protein has been cloned into an expression system, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, insect, plant, and the like.

The present invention's method of imparting stress resistance to plants can involve applying the hypersensitive response elicitor polypeptide or protein in a non-infectious form to all or part of a plant or a plant seed under conditions effective for the elicitor to impart stress resistance. Alternatively, the hypersensitive response elicitor protein or polypeptide can be applied to plants such that seeds recovered from such plants themselves are able to impart stress resistance in plants.

As an alternative to applying a hypersensitive response elicitor polypeptide or protein to plants or plant seeds in order to impart stress resistance in plants or plants grown from the seeds, transgenic plants or plant seeds can be utilized. When utilizing transgenic plants, this involves providing a transgenic plant transformed with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein and growing the plant under conditions effective to permit that DNA molecule to impart stress resistance to plants. Alternatively, a transgenic plant seed transformed with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein can be provided and planted in soil. A plant is then propagated from the planted seed under conditions effective to permit that DNA molecule to impart stress resistance to plants.

The embodiment of the present invention where the hypersensitive response elicitor polypeptide or protein is applied to the plant or plant seed can be carried out in a number of ways, including: 1) application of an isolated hypersensitive response elicitor or 2) application of bacteria which do not cause disease and are transformed with a genes encoding the elicitor. In the latter embodiment, the elicitor can be applied to plants or plant seeds by applying bacteria containing the DNA molecule encoding a hypersensitive response elicitor polypeptide or protein. Such bacteria must be capable of secreting or exporting the elicitor so that the elicitor can contact plant or plant seed cells. In these embodiments, the elicitor is produced by the bacteria *in planta* or on seeds or just prior to introduction of the bacteria to the plants or plant seeds.

The methods of the present invention can be utilized to treat a wide variety of plants or their seeds to impart stress resistance. Suitable plants include dicots and monocots. More particularly, useful crop plants can include: alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean, pea,

chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane. Examples of suitable ornamental plants are: *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

In accordance with the present invention, the term "stress" refers to drought, salt, cold temperatures (e.g., frost), chemical treatment (e.g., insecticides, fungicides, herbicides, fertilizers), water, excessive light, and insufficient light.

The method of the present invention involving application of the hypersensitive response elicitor polypeptide or protein can be carried out through a variety of procedures when all or part of the plant is treated, including leaves, stems, roots, propagules (e.g., cuttings), etc. This may (but need not) involve infiltration of the hypersensitive response elicitor polypeptide or protein into the plant. Suitable application methods include high or low pressure spraying, injection, and leaf abrasion proximate to when elicitor application takes place. When treating plant seeds or propagules (e.g., cuttings), in accordance with the application embodiment of the present invention, the hypersensitive response elicitor protein or polypeptide, in accordance with present invention, can be applied by low or high pressure spraying, coating, immersion, or injection. Other suitable application procedures can be envisioned by those skilled in the art provided they are able to effect contact of the elicitor with cells of the plant or plant seed. Once treated with the hypersensitive response elicitor of the present invention, the seeds can be planted in natural or artificial soil and cultivated using conventional procedures to produce plants. After plants have been propagated from seeds treated in accordance with the present invention, the plants may be treated with one or more applications of the hypersensitive response elicitor protein or polypeptide to impart stress resistance to plants.

The hypersensitive response elicitor polypeptide or protein, in accordance with the present invention, can be applied to plants or plant seeds alone or in a mixture with other materials. Alternatively, the hypersensitive response elicitor

polypeptide or protein can be applied separately to plants with other materials being applied at different times.

A composition suitable for treating plants or plant seeds in accordance with the application embodiment of the present invention contains a hypersensitive response elicitor polypeptide or protein in a carrier. Suitable carriers include water, aqueous solutions, slurries, or dry powders. In this embodiment, the composition contains greater than 500 nM of the elicitor.

Although not required, this composition may contain additional additives including fertilizer, insecticide, fungicide, nematacide, and mixtures thereof. Suitable fertilizers include $(NH_4)_2NO_3$. An example of a suitable insecticide is Malathion. Useful fungicides include Captan.

Other suitable additives include buffering agents, wetting agents, coating agents, and abrading agents. These materials can be used to facilitate the process of the present invention. In addition, the hypersensitive response elicitor can be applied to plant seeds with other conventional seed formulation and treatment materials, including clays and polysaccharides.

In the alternative embodiment of the present invention involving the use of transgenic plants and transgenic seeds, a hypersensitive response elicitor need not be applied topically to the plants or seeds. Instead, transgenic plants transformed with a DNA molecule encoding such an elicitor are produced according to procedures well known in the art.

The vector described above can be microinjected directly into plant cells by use of micropipettes to transfer mechanically the recombinant DNA. Crossway, Mol. Gen. Genetics, 202:179-85 (1985), which is hereby incorporated by reference. The genetic material may also be transferred into the plant cell using polyethylene glycol. Krens, et al., Nature, 296:72-74 (1982), which is hereby incorporated by reference.

Another approach to transforming plant cells with a gene is particle bombardment (also known as biolistic transformation) of the host cell. This can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., which are hereby

incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate th outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the heterologous DNA. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried bacterial cells containing the vector and heterologous DNA) can also be propelled into plant cells.

Yet another method of introduction is fusion of protoplasts with other entities, either minicells, cells, lysosomes, or other fusible lipid-surfaced bodies. Fraley, et al., Proc. Natl. Acad. Sci. USA, 79:1859-63 (1982), which is hereby incorporated by reference.

The DNA molecule may also be introduced into the plant cells by electroporation. Fromm et al., Proc. Natl. Acad. Sci. USA, 82:5824 (1985), which is hereby incorporated by reference. In this technique, plant protoplasts are electroporated in the presence of plasmids containing the expression cassette. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and regenerate.

Another method of introducing the DNA molecule into plant cells is to infect a plant cell with *Agrobacterium tumefaciens* or *A. rhizogenes* previously transformed with the gene. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots or roots, and develop further into plants. Generally, this procedure involves inoculating the plant tissue with a suspension of bacteria and incubating the tissue for 48 to 72 hours on regeneration medium without antibiotics at 25-28°C.

Agrobacterium is a representative genus of the Gram-negative family Rhizobiaceae. Its species are responsible for crown gall (*A. tumefaciens*) and hairy root disease (*A. rhizogenes*). The plant cells in crown gall tumors and hairy roots are induced to produce amino acid derivatives known as opines, which are catabolized only by the bacteria. The bacterial genes responsible for expression of opines are a

convenient source of control elements for chimeric expression cassettes. In addition, assaying for the presence of opines can be used to identify transformed tissue.

Heterologous genetic sequences can be introduced into appropriate plant cells, by means of the Ti plasmid of *A. tumefaciens* or the Ri plasmid of *A. rhizogenes*. The Ti or Ri plasmid is transmitted to plant cells on infection by Agrobacterium and is stably integrated into the plant genome. J. Schell, Science, 237:1176-83 (1987), which is hereby incorporated by reference.

After transformation, the transformed plant cells must be regenerated.

Plant regeneration from cultured protoplasts is described in Evans et al., Handbook of Plant Cell Cultures, Vol. 1: (MacMillan Publishing Co., New York, 1983); and Vasil I.R. (ed.), Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, Orlando, Vol. I, 1984, and Vol. III (1986), which are hereby incorporated by reference.

It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to, all major species of sugarcane, sugar beets, cotton, fruit trees, and legumes.

Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced in the callus tissue. These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is usually reproducible and repeatable.

After the expression cassette is stably incorporated in transgenic plants, it can be transferred to other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

Once transgenic plants of this type are produced, the plants themselves can be cultivated in accordance with conventional procedure with the presence of the

gene encoding the hypersensitive response elicitor resulting in stress resistance to the plant. Alternatively, transgenic seeds or propagules (e.g., cuttings) are recovered from the transgenic plants. The seeds can then be planted in the soil and cultivated using conventional procedures to produce transgenic plants. The transgenic plants are propagated from the planted transgenic seeds under conditions effective to impart stress resistance to plants. While not wishing to be bound by theory, such stress resistance may be RNA mediated or may result from expression of the elicitor polypeptide or protein.

When transgenic plants and plant seeds are used in accordance with the present invention, they additionally can be treated with the same materials as are used to treat the plants and seeds to which a hypersensitive response elicitor in accordance with the present invention is applied. These other materials, including a hypersensitive response elicitor in accordance with the present invention, can be applied to the transgenic plants and plant seeds by the above-noted procedures, including high or low pressure spraying, injection, coating, and immersion. Similarly, after plants have been propagated from the transgenic plant seeds, the plants may be treated with one or more applications of the hypersensitive response elicitor in accordance with the present invention to impart stress resistance. Such plants may also be treated with conventional plant treatment agents (e.g., insecticides, fertilizers, etc.).

EXAMPLES

<u>Example 1</u> - **Hypersensitive Response Elicitor-Treated Cotton is More Resistant to the Damage Caused by Insecticide Stress**

Aphids (*Aphids gossypii*) infect cotton during the entire growth season. The damage of aphid infection ranges from honeydew deposit that contaminates the lint and reduces crop value to defoliation that reduces or destroys crops. To protect plants from aphid infection, cotton is usually sprayed with insecticides, for example Asana XL when the infection pressure is not very high, and Admire when the infestation pressure is high. The effect of a hypersensitive response elicitor on aphids in cotton was studied by a trial involving a randomized complete block design. This

involved treatment with *Erwinia amylovora* hypersensitive response elicitor (i.e. HP-1000™) at 20, 60, and 80 ppm and a chemical insecticide, Asana XL, at 8 oz./ac. Each treatment involved foliar application beginning at cotyledon to three true leaves and thereafter at 14 day intervals using a backpack sprayer. Aphid counts and overall growth of the cotton were made immediately prior to spray application at 14, 28, 35, and 42 days after the first treatment ("DAT 1"). Twenty-five randomly selected leaves per plot were collected at the first three sampling dates and the leaves per plot at the final sampling date.

Results

1. Aphid control: The number of aphids in the hypersensitive response elicitor-treated cotton were significantly reduced in comparison to the chemical treated cotton (see Table 1).

Table 1. Aphid count per leaf on cotton after treatment with Asana XL® or HP-1000™

		Number of aphids per leaf[1]			
		No. sprays applied/days after treatment			
Treatment	Rate[2]	1/14DAT1	2/28DAT1	3/35DAT1	4/42DAT1
Asana XL®	8 oz/ac	0.2 a	32.2 a	110.0 a	546.9 a
HP-1000™	20 μg/ml	0.2 a	7.8 b	22.9 b	322.1 a
HP-1000™	60 μg/ml	0.1 a	4.9 b	34.6 b	168.3 a
HP-1000™	80 μg/ml	0.0 a	2.7 b	25.8 b	510.2 a

[1]Means followed by different letters are significantly different according to Duncan's MRT, P=0.05. [2]Rate for Asana XL® is for formulated product, rate for HP-1000™ is for active ingredient (a.i.).

At 14 days after DAT 1, aphid counts were relatively low across all of the treatments, but by 28 days after DAT 1 (by which time two sprayings had been applied), the number of aphids per leaf were significantly greater in Asana XL-treated plants compared to the hypersensitive response elicitor-treated cottons. By 35 days after DAT 1 (by which time three sprayings had been applied), aphid counts had risen for all treatments, yet aphid counts per leaf were still significantly lower for hypersensitive response elicitor-treated cotton compared to the Asana XL treatment. Finally, at 42 days after DAT 1 (by which time four sprayings had been applied), the number of aphids per leaf had increased to a level that threatened to overwhelm the

plants even when treated with the standard chemical insecticide. To save the trial, another chemical, Pravado (Admire), was applied to all plots to eradicate aphids from the field.

2. Hypersensitive response elicitor-treated cotton was more resistant to the damage caused by Pravado (Admire) and Asana. After the second chemical spraying, it was observed that cotton plants were stress shocked by the insecticides. The cotton plants previously treated with Asana and untreated control were defoliated. On most of the chemical-treated cotton, there were no leaves, or very few leaves, in the lower portion of plants. However, the hypersensitive response elicitor-treated plants, especially the plot where hypersensitive response elicitor was applied at 80 ppm, had no defoliation and the cotton plants were vigorous and healthy. By counting the number of mature balls, it clearly showed that hypersensitive response elicitor-treated plants (at 80 ppm) had more ball setting than chemical and untreated control (Table 2), indicating that hypersensitive response elicitor-treated plants were more tolerant to the stress caused by insecticide.

Table 2. Number of Formed Cotton Balls Counted on Ten Plants in Each of Four Replicates Per Treatment.

Treatment	No. balls/10 plants/replicate
UTC	28
Chemical standard	6
Hypersensitive Response Elicitor	35

Example 2 - Hypersensitive Response Elicitor-Treated Cucumbers are More Resistant to Drought

A cucumber field trial was set up to test the effect of *Erwinia amylovora* hypersensitive response elicitor on disease control, tolerance to drought stress, and yield. Three different rates were tested, there at 15, 30, and 60 μg/ml. In addition to hypersensitive response elicitor treatment, there was an untreated control. Each treatment contained three replicate plots. When the first true leaf emerges, hypersensitive response elicitor was sprayed with a back bag sprayer. The second spray was applied ten days after the first spray. The third application was right after

the recovery of cucumber seedlings after the transplanting to the field. Individual treatment was randomly assigned in the field.

When the first true leaf emerged (Day 0), a first application was sprayed. Usually cucumber seedlings are transplanted when seedlings show two true leaves. It has been known that the recovery rate after the transplanting is closely related to the size of the seedlings. Because of the drought, the seedlings were maintained in the nursery for an extra ten days and the second spray was applied on Day 10. Two days after the second spray, the plants were transplanted into fields and covered with plastic sheets. The plants had 4 – 5 true leaves.

Result

The recovery rate of the transplanted cucumber seedlings was higher for the hypersensitive response elicitor-treated plants than for the untreated control. More than 80% of the hypersensitive response elicitor-treated cucumber seedlings survived, while only 57% untreated plants survived.

Throughout the growth season, there was a serious drought problem. Early field visits indicated that hypersensitive response elicitor-treated plants had more root mass and better over-all growth. Hypersensitive response elicitor-treated cucumber started to flower 14 days earlier than untreated control cucumber. The early flowering resulted in an earlier harvest. In the first harvest, more than 0.4 kilograms of cucumber fruits per plant were harvested from the hypersensitive response elicitor-treated cucumbers; however, virtually no fruit was harvested from untreated control. By the end of the season, untreated plants died due to severe drought, but hypersensitive response elicitor-treated plants were still alive and had one more harvest.

The final yield was significantly different between hypersensitive response elicitor-treated and untreated plants. Hypersensitive response elicitor administered at the rate of 30 ppm produced three times greater yield than the control plants (Table 3).

Table 3. Yield Increase of Cucumber Fruit from Hypersensitive Response Elicitor Treated Plants

Treatment	Replicate	kg/plant	Yield/Replicate		% of the Yield Increase
HP 15	I	1.25	37.5		
	II	1.00	30.0	103.8	241
	III	1.21	36.3		
HP 30	I	1.54	46.2		
	II	1.43	42.9	133.2	339
	III	1.47	44.1		
Control	I	0.43	12.9		
	II	0.41	12.3	39.3	
	III	0.47	14.1		

The increased yield was partially attributed to hypersensitive response elicitor-induced growth enhancement and partially resulted from more tolerance of hypersensitive response elicitor-treated cucumber to drought, because usually the yield increase from hypersensitive response elicitor-induced growth enhancement is between 10-40%.

<u>Example 3</u> - Hypersensitive Response Elicitor-Treated Pepper is More Tolerant to Herbicide Stress

Pepper seedlings were drenched with hypersensitive response elicitor at 20 ppm seven days before transplanting, sprayed seven days after the transplanting, and then, sprayed every fourteen days. Standard chemicals, Brave, Maneb, Kocide, and Admire, were used for the rest of the treatment. In addition to early growth enhancement, which resulted in a higher yield, larger fruit, and resistance to several diseases, hypersensitive response elicitor-treated pepper was more tolerant to herbicide damage. The pepper field was applied with the herbicide SENCOR which is not labeled for pepper. This herbicide is known to cause severe foliar damage to pepper in chemically-treated plants but not with hypersensitive response elicitor-treated plants.

The difference between the adverse effect of the herbicide on the hypersensitive response elicitor and non-hypersensitive response elicitor treated plants is dramatic. See Table 4 below. Thirty-nine of the 60 elicitor-treated plants showed only minor damage by the herbicide, the damaged leaves were less than 20%. In

contrast, 53 out of the 60 chemically-treated pepper plants had severe damage, 40-57% of the leaves were damaged, and 20 plants were dead. The ability of hypersensitive response elicitors to help crops withstand the phytotoxic effects of a herbicide is very important benefit to in agricultural industry.

Table 4. Hypersensitive Response Elicitor-Treated Peppers are More Tolerant to Herbicide Damage.

Treatment		Damage Rating						Damage Index %
		1	2	3	4	5	6	41
Hypersensitive Response Elicitor		1	38	17	3	1	0	
Chemicals	0	1	6	16	19	18		87

Damage Rating: 1. No damage; 2. 0-20% leaves damaged; 3. 20-40% leaves damaged; 4. 40-50% leaves damaged; 6. More than 75% leaves damaged or entire plant dead.

Damage index = sum of each rating times the number of plants under the rating scale, divided by total number of plants times 6.

Damage index for hypersensitive response elicitor-treated plants = $\underline{1x1+2x38+3x17+4x3+5x1+6x0}$ x100% = 41%

Example 4 - Hypersensitive Response Elicitor-Treated Pepper is More Tolerant to Herbicide Stress under Controlled Experimental Conditions

A field trial was conducted to test if hypersensitive elicitor treated pepper would be more tolerant to herbicide stress. The trial contains 6 treatments and 4 replicates for each treatment. The treatments are described as follows:

1. Control, the peppers were neither treated by a hypersensitive response ("HR") elicitor nor by LEXONE™ herbicide (DuPont Agricultural Products, Wilmington, Delaware).

2. Control pepper with application of 0.15 pound LEXONE™ herbicide /acre.

3. Control pepper with application of 0.3 pound LEXONE™ herbicide /acre.

4. HR elicitor treatment with no application of LEXONE™ herbicide using a formulated product known as MESSENGER™ biopesticide (Eden Bioscience Corporation, Bothell, Washington) containing 3% HR elicitor protein was used.

5. HR elicitor treatment with application of 0.15 pound LEXONE™ herbicide /acre.

6. HR elicitor treatment with application of 0.3 pound LEXONE™ herbicide /acre.

LEXONE™ contains the same active ingredient as SENCOR™ herbicide (Bayer, Kansas City, Missouri) used in Example 3. Pepper seedlings were drenched with MESSENGER™ solution at the concentration of HR elicitor protein of about 20 ppm seven days before transplanting into the field and then sprayed every 14 days after the transplanting. LEXONE was applied at high (0.3 pound/acre) and low levels (0.15 pound/acre). 50 gallon water and 100 mL of the herbicide solution was introduced into the root zone of each plant in the respective treatment five weeks after transplant into the field.

The treatments were evaluated for the percent of chlorosis caused by the LEXONE™ herbicide application and for the pepper yield. HR elicitor-treated plants exposed to the high rate of herbicide had significantly less chlorosis and produced 108 % more fruit in comparison to the non-hypersensitive response elicitor treated plants exposed to the same amount of herbicide. See Tables 5 and 6 below. There was no significant difference in the reduction of chlorosis at the low rate of herbicide between the HR elicitor treated and non-HR elicitor treated peppers. However, the HR elicitor treated plants produced 15% more fruit than the corresponding control plants exposed to the same amount of herbicide. There was no chlorosis in either the check or HR elicitor-treated plants that did not receive LEXONE™ herbicide treatment.

The HR elicitor treated plants were much less severely affected by the herbicide application than the respective control plants at the high rate of herbicide. However, the amount of visual chlorosis was similar at the low rate for both the check and HR elicitor-treated plants. More importantly, the yields from both the high and low rate herbicide treatments of HR elicitor treated plants were less severely effected

by the herbicide than the checks. These findings further confirm that HR elicitors can help crops withstand the phytotoxic effects of herbicides and are very beneficial to the agricultural industry.

Table 5. Reduction of Foliar Chlorosis and Increase in Yield in Hypersensitive Response Elicitor Treated Plants after Exposure to LEXONE™ Herbicide

		Percent foliar chlorosis and yield of pepper					
Treatment	A	B	C	D	E	Yield (pound)	% difference from the respective control
6 (MESSENGER™+ High rate LEXONE™)	13.75	30.00	37.50	36.25	40.00	8.31	108 %
3 (High rate LEXONE™)	26.25	43.75	51.25	50.00	51.25	4.00	-
5 (MESSENGER™ + low rate LENOXE™)	16.25	22.50	28.75	23.75	27.50	8.00	15 %
2 (LENOXE™)	12.50	20.00	25.00	25.00	23.75	6.81	-

Table 6. Weight of Harvested Peppers Increased in Hypersensitive Response Elicitor Treated Plants after Exposure to LEXONE™ Herbicide Compared to Check Plants.

Treatment	Weight of peppers harvested 12/1/98 in pounds
HP20 + high rate LEXONE™	8.31
Check + high rate LEXONE™	4.00
HP20 + low rate LEXONE™	8.00
Check + low rate LEXONE™	6.81

Example 5 - Hypersensitive Response Elicitor-Treated Cotton is More Tolerant to Drought Stress

A non-irrigated cotton trial experienced 26 consecutive days of drought. The average daily heat index was near or over 100 degrees F, adding to the stress placed on the plants in the field.

Observations in the field indicated that plants treated with HR elicitor at the concentration of 15 ppm (2.2 oz formulated product, MESSENGER™ containing 3 % active ingredient HR elicitor protein) were more vigorous and had less defoliation than the check plants as a result of the heat and drought stress. Equal numbers of plants from the MESSENGER™-treated and the non-MESSENGER™ treated plots were carefully removed from the field and mapped for the number of nodes and bolls by position. The plants were also weighed on a Metler analytical scale to determine whole plant, root and shoot weights.

MESSENGER™ treated plants survived the heat and drought stresses much better than the untreated plants did. Plants treated with MESSENGER™ had 37.6% more root and shoot mass than the check plants (Table 7). The MESSENGER™ treated plants also had significantly more cotton bolls than the check plants (Table 8). The number of cotton bolls from positions 1 and 2 have a significant contribution to the overall yield. Table 8 showed that MESSENGER™ treated plants had 47% more bolls in positions 1 and 2 and 57% more boll from a whole plant in comparison to the yield achieved using a grower standard treatment (i.e. with no MESSENGER™ treatment). A common reaction to stress in cotton is for the plant to abort bolls. The results indicate that MESSENGER™-treated plants are more tolerant to the drought stress.

Table 7. Weight per Plant of Non-Irrigated Cotton Following 26 Days of Drought.

Treatment	Root weight (pond/plant)	%Difference	Shoot weight (pond/plant)	% difference	Whole plant weight (pond/plant)	% difference
MESSENGER™ 2.2 oz/acre	0.041 a*	37.6 %	0.505 a	37.5 %	0.546	37.5 %
Control (Grower standard)	0.0298 b		0.367 b		0.397	
Level of statistically significant	P=0.119		P=0.034			P=0.033

* Same letter indicates no statistical difference between the two treatments at the defined level; different letter indicates a statistical difference between the two treatments at the defined level.

Table 8. Number of Bolls per 5 Plants at the Number 1 & 2 positions, and Total Number of Bolls from Whole Plants in Non-irrigated Cotton Following 26 days of drought.

Treatment	Avg. # bolls in the #1 & 2 position	Percent difference	Avg. # of total bolls per 5 plant	Percent difference
MESSENGER™ 2.2 OZ.	18.4 a	+46.0%	21.4 a	+57.0%
Check	12.6 b		13.6 b	-
Statistically significant level	P=0.032		P=0.01	

* Same letter indicates no statistical difference between the two treatments at the defined level; different letter indicates a statistical difference between the two treatments at the defined level.

<u>Example 6</u> - Hypersensitive Response Elicitor-Treated Tomato is More Tolerant to Calcium Deficiency

Calcium is an important element for plant physiology and development. A deficiency in calcium can cause several plant diseases. For example, blossom-end rot is caused by a localized calcium deficiency in the distal end of the tomato fruit. Because calcium is not a highly mobile element, a deficiency can occur with a fluctuation in water supply. In the past, tomato growers experienced higher level of blossom-end rot during dry weather conditions when infrequent rains storms dumped a lot of water and then return to a hot and dry condition quickly. Lowering or raising the irrigation water table erratically during a dry and hot growing season can also increase the disease.

A field trial was designed to test if HR elicitor protein-treated tomato can be more tolerant to the calcium deficiency under a dry hot growing season. MESSENGER™, the formulated product containing 3% HR elicitor, was used for the trial. The application rate of the MESSENGER™ was 2.27 oz per care. The first spray of MESSENGER™ was carried out 7 days before the transplanting and then every 14-days after transplanting. MESSENGER™-treated tomatoes were compared with a standard grower treatment not utilizing MESSENGER™. Each treatment had 4 replicates.

The number of infected fruit was counted from a 100 square foot field. The rot typically begins with light tan water soaked lesions, which then enlarge, and then turn black. In a survey, about 20% of the fruits were infected. Severe end-rot

symptoms occurred in the standard treatment; however, an average of only 2.5 % of the fruit was infected in the MESSENGER™-treated plants. The harvest data showed that MESSENGER™-treated plants had 8% more marketable fruit (Table 9). The test results demonstrated that MESSENGER™-treatment can reduce the stress resulting from calcium deficiency and increase plant resistance to blossom-end rot.

Table 9. Hypersensitive Response Elicitor Treatment Reduced Blossom-End Rot Infection, Increased Yield of Tomato Fruit

Treatment	Blossom-End Infected Fruit*				Tomato Fruit Yield	
	Rep I	Rep II	Rep III	Rep IV	Bin/Acre	% Difference
MESSENGER™	0	9	0	1	35	8
Standard Treatment)	24	22	16	17	31.5	-

*The data were collected from the fruits in 100 square foot plot

Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

WHAT IS CLAIMED:

1. A method of imparting stress resistance to plants comprising: applying a hypersensitive response elicitor protein or polypeptide in a non-infectious form to a plant or plant seed under conditions effective to impart stress resistance.

2. A method according to claim 1, wherein the stress resistance is resistance to a stress selected from the group consisting of climated related stress, air pollution stress, chemical stress, and nutritional stress.

3. A method according to claim 2, wherein the stress is chemical stress where the chemical is selected from the group consisting of insecticides, fungicides, herbicides, and heavy metals.

4. A method according to claim 2, wherein the stress is climate-related stress selected from the group consisting of drought, water, frost, cold temperature, high temperature, excessive light, and insufficient light.

5. A method according to claim 2, wherein the stress is air pollution stress selected from the group consisting of carbon dioxide, carbon monoxide, sulfur dioxide, NO_x, hydrocarbons, ozone, ultraviolet radiation, and acidic rain.

6. A method according to claim 2, wherein the stress is nutritional stress where the nutritional stress is caused by fertilizer, micronutrients, or macronutrients.

7. A method according to claim 1, wherein the hypersensitive response elicitor protein or polypeptide is derived from *Erwinia*, *Pseudomonas*, *Xanthamonas*, *Phythophthera*, or *Clavibacter*.

8. A method according to claim 7, wherein the hypersensitive response elicitor protein or polypeptide is derived from *Erwinia amylovora*, *Erwinia carotovora*, *Erwinia chrysanthemi*, and *Erwinia stewartii*.

9. A method according to claim 7, wherein the hypersensitive response elicitor protein or polypeptide is derived from *Pseudomonas syringae* or *Pseudomonas solancearum*.

10. A method according to claim 7, wherein the hypersensitive response elicitor protein or polypeptide is derived from a *Xanthamonas* species.

11. A method according to claim 7, wherein the hypersensitive response elicitor protein or polypeptide is derived from a *Phythophthera*.

12. A method according to claim 7, wherein the hypersensitive response elicitor protein or polypeptide is derived from *Clavibacter michiganesis* subsp. *sepedonicus*.

13. A method according to claim 1, wherein plants are treated during said applying.

14. A method according to claim 1, wherein plant seeds are treated during said applying, said method further comprising:

planting the seeds treated with the hypersensitive response elicitor protein or polypeptide in natural or artificial soil and

propagating plants from seeds planted in soil.

15. A method according to claim 1, wherein the plant is selected from the group consisting of alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear,

melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

16. A method according to claim 1, wherein the plant is selected from the group consisting of *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

17. A method of imparting stress resistance to plants comprising:

providing a transgenic plant or plant seed transformed with a DNA molecule which encodes for a hypersensitive response elicitor protein or polypeptide and

growing the transgenic plant or plants produced from the transgenic plant seeds under conditions effective to impart stress resistance.

18. A method according to claim 17, wherein a transgenic plant is provided.

19. A method according to claim 17, wherein a transgenic plant seed is provided, said method further comprising:

planting the transgenic seeds in natural or artificial soil and propagating plants from seeds planted in soil..

20. A method according to claim 17, wherein the stress resistance is resistance to a stress selected from the group consisting of climated related stress, air pollution stress, chemical stress, and nutritional stress.

21. A method according to claim 20, wherein the stress is chemical stress where the chemical is selected from the group consisting of insecticides, fungicides, herbicides, and heavy metals.

22. A method according to claim 20, wherein the stress is climate-related stress selected from the group consisting of drought, water, frost, cold temperature, high temperature, excessive light, and insufficient light.

23. A method according to claim 20, wherein the stress is air pollution stress selected from the group consisting of carbon dioxide, carbon monoxide, sulfur dioxide, NO_x, hydrocarbons, ozone, ultraviolet radiation, and acidic rain.

24. A method according to claim 20, wherein the stress is nutritional stress where the nutritional stress is caused by fertilizer, micronutrients, or macronutrients.

25. A method according to claim 20, wherein the hypersensitive response elicitor protein or polypeptide is derived from *Erwinia*, *Pseudomonas*, *Xanthamonas*, *Phythophthera*, or *Clavibacter*.

26. A method according to claim 25, wherein thc hypersensitive response elicitor protein or polypeptide is derived from *Erwinia amylovora*, *Erwinia carotovora*, *Erwinia chrysanthemi*, and *Erwinia stewartii*.

27. A method according to claim 25, wherein the hypersensitive response elicitor protein or polypeptide is derived from *Pseudomonas syringae* or *Pseudomonas solancearum*.

28. A method according to claim 25, wherein the hypersensitive response elicitor protein or polypeptide is derived from a *Xanthamonas* species.

29. A method according to claim 20, wherein the plant is selected from the group consisting of alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic,

eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

30. A method according to claim 20, wherein the plant is selected from the group consisting of *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

SEQUENCE LISTING

<110> Eden Bioscience Corporation

<120> HYPERSENSITIVE RESPONSE ELICITOR-INDUCED STRESS
RESISTANCE

<130> 21829/42

<140>
<141>

<150> 60/107,243
<151> 1998-11-05

<160> 18

<170> PatentIn Ver. 2.0

<210> 1
<211> 338
<212> PRT
<213> Erwinia chrysanthemi

<400> 1
```
Met Gln Ile Thr Ile Lys Ala His Ile Gly Gly Asp Leu Gly Val Ser
  1               5                  10                  15

Gly Leu Gly Ala Gln Gly Leu Lys Gly Leu Asn Ser Ala Ala Ser Ser
             20                  25                  30

Leu Gly Ser Ser Val Asp Lys Leu Ser Ser Thr Ile Asp Lys Leu Thr
         35                  40                  45

Ser Ala Leu Thr Ser Met Met Phe Gly Gly Ala Leu Ala Gln Gly Leu
     50                  55                  60

Gly Ala Ser Ser Lys Gly Leu Gly Met Ser Asn Gln Leu Gly Gln Ser
 65                  70                  75                  80

Phe Gly Asn Gly Ala Gln Gly Ala Ser Asn Leu Leu Ser Val Pro Lys
                 85                  90                  95

Ser Gly Gly Asp Ala Leu Ser Lys Met Phe Asp Lys Ala Leu Asp Asp
            100                 105                 110

Leu Leu Gly His Asp Thr Val Thr Lys Leu Thr Asn Gln Ser Asn Gln
        115                 120                 125
```

Leu Ala Asn Ser Met Leu Asn Ala Ser Gln Met Thr Gln Gly Asn Met
130 135 140

Asn Ala Phe Gly Ser Gly Val Asn Asn Ala Leu Ser Ser Ile Leu Gly
145 150 155 160

Asn Gly Leu Gly Gln Ser Met Ser Gly Phe Ser Gln Pro Ser Leu Gly
165 170 175

Ala Gly Gly Leu Gln Gly Leu Ser Gly Ala Gly Ala Phe Asn Gln Leu
180 185 190

Gly Asn Ala Ile Gly Met Gly Val Gly Gln Asn Ala Ala Leu Ser Ala
195 200 205

Leu Ser Asn Val Ser Thr His Val Asp Gly Asn Asn Arg His Phe Val
210 215 220

Asp Lys Glu Asp Arg Gly Met Ala Lys Glu Ile Gly Gln Phe Met Asp
225 230 235 240

Gln Tyr Pro Glu Ile Phe Gly Lys Pro Glu Tyr Gln Lys Asp Gly Trp
245 250 255

Ser Ser Pro Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser Lys
260 265 270

Pro Asp Asp Asp Gly Met Thr Gly Ala Ser Met Asp Lys Phe Arg Gln
275 280 285

Ala Met Gly Met Ile Lys Ser Ala Val Ala Gly Asp Thr Gly Asn Thr
290 295 300

Asn Leu Asn Leu Arg Gly Ala Gly Gly Ala Ser Leu Gly Ile Asp Ala
305 310 315 320

Ala Val Val Gly Asp Lys Ile Ala Asn Met Ser Leu Gly Lys Leu Ala
325 330 335

Asn Ala

<210> 2
<211> 2141
<212> DNA
<213> Erwinia chrysanthemi

<400> 2

```
cgattttacc cgggtgaacg tgctatgacc gacagcatca cggtattcga caccgttacg 60
gcgtttatgg ccgcgatgaa ccggcatcag gcggcgcgct ggtcgccgca atccggcgtc 120
gatctggtat ttcagtttgg ggacaccggg cgtgaactca tgatgcagat tcagccgggg 180
cagcaatatc ccggcatgtt gcgcacgctg ctcgctcgtc gttatcagca ggcggcagag 240
tgcgatggct gccatctgtg cctgaacggc agcgatgtat tgatcctctg gtggccgctg 300
ccgtcggatc ccggcagtta tccgcaggtg atcgaacgtt tgtttgaact ggcgggaatg 360
acgttgccgt cgctatccat agcaccgacg gcgcgtccgc agacagggaa cggacgcgcc 420
cgatcattaa gataaaggcg gctttttta ttgcaaaacg gtaacggtga ggaaccgttt 480
caccgtcggc gtcactcagt aacaagtatc catcatgatg cctacatcgg gatcggcgtg 540
ggcatccgtt gcagatactt ttgcgaacac ctgacatgaa tgaggaaacg aaattatgca 600
aattacgatc aaagcgcaca tcggcggtga tttgggcgtc tccggtctgg ggctgggtgc 660
tcagggactg aaaggactga attccgcggc ttcatcgctg ggttccagcg tggataaact 720
gagcagcacc atcgataagt tgacctccgc gctgacttcg atgatgtttg gcggcgcgct 780
ggcgcagggg ctgggcgcca gctcgaaggg gctggggatg agcaatcaac tgggccagtc 840
tttcggcaat ggcgcgcagg gtgcgagcaa cctgctatcc gtaccgaaat ccggcggcga 900
tgcgttgtca aaaatgtttg ataaagcgct ggacgatctg ctgggtcatg acaccgtgac 960
caagctgact aaccagagca accaactggc taattcaatg ctgaacgcca gccagatgac 1020
ccagggtaat atgaatgcgt tcggcagcgg tgtgaacaac gcactgtcgt ccattctcgg 1080
caacggtctc ggccagtcga tgagtggctt ctctcagcct tctctggggg caggcggctt 1140
gcagggcctg agcggcgcgg gtgcattcaa ccagttgggt aatgccatcg gcatgggcgt 1200
ggggcagaat gctgcgctga gtgcgttgag taacgtcagc acccacgtag acggtaacaa 1260
ccgccacttt gtagataaag aagatcgcgg catggcgaaa gagatcggcc agtttatgga 1320
tcagtatccg gaaatattcg gtaaaccgga ataccagaaa gatggctgga gttcgccgaa 1380
gacggacgac aaatcctggg ctaaagcgct gagtaaaccg gatgatgacg gtatgaccgg 1440
cgccagcatg gacaaattcc gtcaggcgat gggtatgatc aaaagcgcgg tggcgggtga 1500
taccggcaat accaacctga acctgcgtgg cgcgggcggt gcatcgctgg gtatcgatgc 1560
ggctgtcgtc ggcgataaaa tagccaacat gtcgctgggt aagctggcca acgcctgata 1620
atctgtgctg gcctgataaa gcggaaacga aaaaagagac ggggaagcct gtctcttttc 1680
ttattatgcg gtttatgcgg ttacctggac cggttaatca tcgtcatcga tctggtacaa 1740
acgcacattt tcccgttcat tcgcgtcgtt acgcgccaca atcgcgatgg catcttcctc 1800
gtcgctcaga ttgcgcggct gatggggaac gccgggtgga atatagagaa actcgccggc 1860
cagatggaga cacgtctgcg ataaatctgt gccgtaacgt gtttctatcc gcccctttag 1920
cagatagatt gcggtttcgt aatcaacatg gtaatgcggt tccgcctgtg cgccggccgg 1980
gatcaccaca atattcatag aaagctgtct tgcacctacc gtatcgcggg agataccgac 2040
aaaatagggc agttttgcg tggtatccgt ggggtgttcc ggcctgacaa tcttgagttg 2100
gttcgtcatc atctttctcc atctgggcga cctgatcggt t 2141
```

<210> 3
<211> 403
<212> PRT
<213> Erwinia amylovora

<400> 3

```
Met Ser Leu Asn Thr Ser Gly Leu Gly Ala Ser Thr Met Gln Ile Ser
 1               5                   10                  15
```

Ile Gly Gly Ala Gly Gly Asn Asn Gly Leu Leu Gly Thr Ser Arg Gln
20 25 30

Asn Ala Gly Leu Gly Gly Asn Ser Ala Leu Gly Leu Gly Gly Gly Asn
35 40 45

Gln Asn Asp Thr Val Asn Gln Leu Ala Gly Leu Leu Thr Gly Met Met
50 55 60

Met Met Met Ser Met Met Gly Gly Gly Gly Leu Met Gly Gly Gly Leu
65 70 75 80

Gly Gly Gly Leu Gly Asn Gly Leu Gly Gly Ser Gly Gly Leu Gly Glu
85 90 95

Gly Leu Ser Asn Ala Leu Asn Asp Met Leu Gly Gly Ser Leu Asn Thr
100 105 110

Leu Gly Ser Lys Gly Gly Asn Asn Thr Thr Ser Thr Thr Asn Ser Pro
115 120 125

Leu Asp Gln Ala Leu Gly Ile Asn Ser Thr Ser Gln Asn Asp Asp Ser
130 135 140

Thr Ser Gly Thr Asp Ser Thr Ser Asp Ser Ser Asp Pro Met Gln Gln
145 150 155 160

Leu Leu Lys Met Phe Ser Glu Ile Met Gln Ser Leu Phe Gly Asp Gly
165 170 175

Gln Asp Gly Thr Gln Gly Ser Ser Ser Gly Gly Lys Gln Pro Thr Glu
180 185 190

Gly Glu Gln Asn Ala Tyr Lys Lys Gly Val Thr Asp Ala Leu Ser Gly
195 200 205

Leu Met Gly Asn Gly Leu Ser Gln Leu Leu Gly Asn Gly Gly Leu Gly
210 215 220

Gly Gly Gln Gly Gly Asn Ala Gly Thr Gly Leu Asp Gly Ser Ser Leu
225 230 235 240

Gly Gly Lys Gly Leu Gln Asn Leu Ser Gly Pro Val Asp Tyr Gln Gln
245 250 255

Leu Gly Asn Ala Val Gly Thr Gly Ile Gly Met Lys Ala Gly Ile Gln
260 265 270

Ala Leu Asn Asp Ile Gly Thr His Arg His Ser Ser Thr Arg Ser Phe
275 280 285

Val Asn Lys Gly Asp Arg Ala Met Ala Lys Glu Ile Gly Gln Phe Met
290 295 300

Asp Gln Tyr Pro Glu Val Phe Gly Lys Pro Gln Tyr Gln Lys Gly Pro
305 310 315 320

Gly Gln Glu Val Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser
325 330 335

Lys Pro Asp Asp Asp Gly Met Thr Pro Ala Ser Met Glu Gln Phe Asn
340 345 350

Lys Ala Lys Gly Met Ile Lys Arg Pro Met Ala Gly Asp Thr Gly Asn
355 360 365

Gly Asn Leu Gln Ala Arg Gly Ala Gly Gly Ser Ser Leu Gly Ile Asp
370 375 380

Ala Met Met Ala Gly Asp Ala Ile Asn Asn Met Ala Leu Gly Lys Leu
385 390 395 400

Gly Ala Ala

<210> 4
<211> 1288
<212> DNA
<213> Erwinia amylovora

<400> 4
aagcttcggc atggcacgtt tgaccgttgg gtcggcaggg tacgtttgaa ttattcataa 60
gaggaatacg ttatgagtct gaatacaagt gggctgggag cgtcaacgat gcaaatttct 120
atcggcggtg cgggcggaaa taacgggttg ctgggtacca gtcgccagaa tgctgggttg 180
ggtggcaatt ctgcactggg gctgggcggc ggtaatcaaa atgataccgt caatcagctg 240
gctggcttac tcaccggcat gatgatgatg atgagcatga tgggcggtgg tgggctgatg 300
ggcggtggct taggcggtgg cttaggtaat ggcttgggtg gctcaggtgg cctgggcgaa 360
ggactgtcga acgcgctgaa cgatatgtta ggcggttcgc tgaacacgct gggctcgaaa 420
ggcggcaaca ataccacttc aacaacaaat tccccgctgg accaggcgct gggtattaac 480
tcaacgtccc aaaacgacga ttccacctcc ggcacagatt ccacctcaga ctccagcgac 540
ccgatgcagc agctgctgaa gatgttcagc gagataatgc aaagcctgtt tggtgatggg 600
caagatggca cccagggcag ttcctctggg ggcaagcagc cgaccgaagg cgagcagaac 660
gcctataaaa aaggagtcac tgatgcgctg tcgggcctga tgggtaatgg tctgagccag 720
ctccttggca acgggggact gggaggtggt cagggcggta atgctggcac gggtcttgac 780
ggttcgtcgc tgggcggcaa agggctgcaa aacctgagcg ggccggtgga ctaccagcag 840

ttaggtaacg ccgtgggtac cggtatcggt atgaaagcgg gcattcaggc gctgaatgat 900
atcggtacgc acaggcacag ttcaacccgt tctttcgtca ataaaggcga tcgggcgatg 960
gcgaaggaaa tcggtcagtt catggaccag tatcctgagg tgtttggcaa gccgcagtac 1020
cagaaaggcc cgggtcagga ggtgaaaacc gatgacaaat catgggcaaa agcactgagc 1080
aagccagatg acgacggaat gacaccagcc agtatggagc agttcaacaa agccaagggc 1140
atgatcaaaa ggcccatggc gggtgatacc ggcaacggca acctgcaggc acgcggtgcc 1200
ggtggttctt cgctgggtat tgatgccatg atggccggtg atgccattaa caatatggca 1260
cttggcaagc tgggcgcggc ttaagctt 1288

<210> 5
<211> 1344
<212> DNA
<213> Erwinia amylovora

<400> 5
atgtcaattc ttacgcttaa caacaatacc tcgtcctcgc cgggtctgtt ccagtccggg 60
ggggacaacg ggcttggtgg tcataatgca aattctgcgt tggggcaaca acccatcgat 120
cggcaaacca ttgagcaaat ggctcaatta ttggcggaac tgttaaagtc actgctatcg 180
ccacaatcag gtaatgcggc aaccggagcc ggtggcaatg accagactac aggagttggt 240
aacgctggcg gcctgaacgg acgaaaaggc acagcaggaa ccactccgca gtctgacagt 300
cagaacatgc tgagtgagat gggcaacaac gggctggatc aggccatcac gcccgatggc 360
cagggcggcg ggcagatcgg cgataatcct ttactgaaag ccatgctgaa gcttattgca 420
cgcatgatgg acggccaaag cgatcagttt ggccaacctg gtacgggcaa caacagtgcc 480
tcttccggta cttcttcatc tggcggttcc ccttttaacg atctatcagg ggggaaggcc 540
ccttccggca actccccttc cggcaactac tctcccgtca gtaccttctc acccccatcc 600
acgccaacgt cccctacctc accgcttgat ttcccttctt ctcccaccaa agcagccggg 660
ggcagcacgc cggtaaccga tcatcctgac cctgttggta gcgcgggcat cggggccgga 720
aattcggtgg ccttcaccag cgccggcgct aatcagacgg tgctgcatga caccattacc 780
gtgaaagcgg gtcaggtgtt tgatggcaaa ggacaaacct tcaccgccgg ttcagaatta 840
ggcgatggcg gccagtctga aaaccagaaa ccgctgttta tactggaaga cggtgccagc 900
ctgaaaaacg tcaccatggg cgacgacggg gcggatggta ttcatcttta cggtgatgcc 960
aaaatagaca atctgcacgt caccaacgtg ggtgaggacg cgattaccgt taagccaaac 1020
agcgcgggca aaaaatccca cgttgaaatc actaacagtt ccttcgagca cgcctctgac 1080
aagatcctgc agctgaatgc cgatactaac ctgagcgttg acaacgtgaa ggccaaagac 1140
tttggtactt ttgtacgcac taacggcggt caacagggta actgggatct gaatctgagc 1200
catatcagcg cagaagacgg taagttctcg ttcgttaaaa gcgatagcga ggggctaaac 1260
gtcaatacca gtgatatctc actgggtgat gttgaaaacc actacaaagt gccgatgtcc 1320
gccaacctga aggtggctga atga 1344

<210> 6
<211> 447
<212> PRT
<213> Erwinia amylovora

<400> 6
Met Ser Ile Leu Thr Leu Asn Asn Asn Thr Ser Ser Ser Pro Gly Leu
1 5 10 15

Phe Gln Ser Gly Gly Asp Asn Gly Leu Gly Gly His Asn Ala Asn Ser
20 25 30

Ala Leu Gly Gln Gln Pro Ile Asp Arg Gln Thr Ile Glu Gln Met Ala
35 40 45

Gln Leu Leu Ala Glu Leu Leu Lys Ser Leu Leu Ser Pro Gln Ser Gly
50 55 60

Asn Ala Ala Thr Gly Ala Gly Gly Asn Asp Gln Thr Thr Gly Val Gly
65 70 75 80

Asn Ala Gly Gly Leu Asn Gly Arg Lys Gly Thr Ala Gly Thr Thr Pro
85 90 95

Gln Ser Asp Ser Gln Asn Met Leu Ser Glu Met Gly Asn Asn Gly Leu
100 105 110

Asp Gln Ala Ile Thr Pro Asp Gly Gln Gly Gly Gly Gln Ile Gly Asp
115 120 125

Asn Pro Leu Leu Lys Ala Met Leu Lys Leu Ile Ala Arg Met Met Asp
130 135 140

Gly Gln Ser Asp Gln Phe Gly Gln Pro Gly Thr Gly Asn Asn Ser Ala
145 150 155 160

Ser Ser Gly Thr Ser Ser Ser Gly Gly Ser Pro Phe Asn Asp Leu Ser
165 170 175

Gly Gly Lys Ala Pro Ser Gly Asn Ser Pro Ser Gly Asn Tyr Ser Pro
180 185 190

Val Ser Thr Phe Ser Pro Pro Ser Thr Pro Thr Ser Pro Thr Ser Pro
195 200 205

Leu Asp Phe Pro Ser Ser Pro Thr Lys Ala Ala Gly Gly Ser Thr Pro
210 215 220

Val Thr Asp His Pro Asp Pro Val Gly Ser Ala Gly Ile Gly Ala Gly
225 230 235 240

Asn Ser Val Ala Phe Thr Ser Ala Gly Ala Asn Gln Thr Val Leu His
245 250 255

Asp Thr Ile Thr Val Lys Ala Gly Gln Val Phe Asp Gly Lys Gly Gln
260 265 270

Thr Phe Thr Ala Gly Ser Glu Leu Gly Asp Gly Gly Gln Ser Glu Asn
275 280 285

Gln Lys Pro Leu Phe Ile Leu Glu Asp Gly Ala Ser Leu Lys Asn Val
290 295 300

Thr Met Gly Asp Asp Gly Ala Asp Gly Ile His Leu Tyr Gly Asp Ala
305 310 315 320

Lys Ile Asp Asn Leu His Val Thr Asn Val Gly Glu Asp Ala Ile Thr
325 330 335

Val Lys Pro Asn Ser Ala Gly Lys Lys Ser His Val Glu Ile Thr Asn
340 345 350

Ser Ser Phe Glu His Ala Ser Asp Lys Ile Leu Gln Leu Asn Ala Asp
355 360 365

Thr Asn Leu Ser Val Asp Asn Val Lys Ala Lys Asp Phe Gly Thr Phe
370 375 380

Val Arg Thr Asn Gly Gly Gln Gln Gly Asn Trp Asp Leu Asn Leu Ser
385 390 395 400

His Ile Ser Ala Glu Asp Gly Lys Phe Ser Phe Val Lys Ser Asp Ser
405 410 415

Glu Gly Leu Asn Val Asn Thr Ser Asp Ile Ser Leu Gly Asp Val Glu
420 425 430

Asn His Tyr Lys Val Pro Met Ser Ala Asn Leu Lys Val Ala Glu
435 440 445

<210> 7
<211> 5517
<212> DNA
<213> Erwinia amylovora

<400> 7
atggaattaa aatcactggg aactgaacac aaggcggcag tacacacagc ggcgcacaac 60
cctgtggggc atggtgttgc cttacagcag ggcagcagca gcagcagccc gcaaaatgcc 120
gctgcatcat tggcggcaga aggcaaaaat cgtgggaaaa tgccgagaat tcaccagcca 180
tctactgcgg ctgatggtat cagcgctgct caccagcaaa agaaatcctt cagtctcagg 240
ggctgtttgg ggacgaaaaa attttccaga tcggcaccgc agggccagcc aggtaccacc 300
cacagcaaag gggcaacatt gcgcgatctg ctggcgcggg acgacggcga aacgcagcat 360
gaggcggccg cgccagatgc ggcgcgtttg acccgttcgg gcggcgtcaa acgccgcaat 420
atggacgaca tggccgggcg gccaatggtg aaaggtggca gcggcgaaga taaggtacca 480

acgcagcaaa aacggcatca gctgaacaat tttggccaga tgcgccaaac gatgttgagc 540
aaaatggctc acccggcttc agccaacgcc ggcgatcgcc tgcagcattc accgccgcac 600
atcccgggta gccaccacga aatcaaggaa gaaccggttg gctccaccag caaggcaaca 660
acggcccacg cagacagagt ggaaatcgct caggaagatg acgacagcga attccagcaa 720
ctgcatcaac agcggctggc gcgcgaacgg gaaaatccac cgcagccgcc caaactcggc 780
gttgccacac cgattagcgc caggtttcag cccaaactga ctgcggttgc ggaaagcgtc 840
cttgagggga cagataccac gcagtcaccc cttaagccgc aatcaatgct gaaaggaagt 900
ggagccgggg taacgccgct ggcggtaacg ctggataaag gcaagttgca gctggcaccg 960
gataatccac ccgcgctcaa tacgttgttg aagcagacat tgggtaaaga cacccagcac 1020
tatctggcgc accatgccag cagcgacggt agccagcatc tgctgctgga caacaaaggc 1080
cacctgtttg atatcaaaag caccgccacc agctatagcg tgctgcacaa cagccacccc 1140
ggtgagataa agggcaagct ggcgcaggcg ggtactggct ccgtcagcgt agacggtaaa 1200
agcggcaaga tctcgctggg gagcggtacg caaagtcaca acaaaacaat gctaagccaa 1260
ccgggggaag cgcaccgttc cttattaacc ggcatttggc agcatcctgc tggcgcagcg 1320
cggccgcagg gcgagtcaat ccgcctgcat gacgacaaaa ttcatatcct gcatccggag 1380
ctgggcgtat ggcaatctgc ggataaagat acccacagcc agctgtctcg ccaggcagac 1440
ggtaagctct atgcgctgaa agacaaccgt accctgcaaa acctctccga taataaatcc 1500
tcagaaaagc tggtcgataa aatcaaatcg tattccgttg atcagcgggg gcaggtggcg 1560
atcctgacgg atactcccgg ccgccataag atgagtatta tgccctcgct ggatgcttcc 1620
ccggagagcc atatttccct cagcctgcat tttgccgatg cccaccaggg gttattgcac 1680
gggaagtcgg agcttgaggc acaatctgtc gcgatcagcc atgggcgact ggttgtggcc 1740
gatagcgaag gcaagctgtt tagcgccgcc attccgaagc aaggggatgg aaacgaactg 1800
aaaatgaaag ccatgcctca gcatgcgctc gatgaacatt ttggtcatga ccaccagatt 1860
tctggatttt tccatgacga ccacggccag cttaatgcgc tggtgaaaaa taacttcagg 1920
cagcagcatg cctgcccgtt gggtaacgat catcagtttc accccggctg gaacctgact 1980
gatgcgctgg ttatcgacaa tcagctgggg ctgcatcata ccaatcctga accgcatgag 2040
attcttgata tggggcattt aggcagcctg gcgttacagg agggcaagct tcactatttt 2100
gaccagctga ccaaagggtg gactggcgcg gagtcagatt gtaagcagct gaaaaaaggc 2160
ctggatggag cagcttatct actgaaagac ggtgaagtga aacgcctgaa tattaatcag 2220
agcacctcct ctatcaagca cggaacggaa aacgtttttt cgctgccgca tgtgcgcaat 2280
aaaccggagc cgggagatgc cctgcaaggg ctgaataaag acgataaggc ccaggccatg 2340
gcggtgattg ggtaaataa atacctggcg ctgacggaaa aaggggacat tcgctccttc 2400
cagataaaac ccggcaccca gcagttggag cggccggcac aaactctcag ccgcgaaggt 2460
atcagcggcg aactgaaaga cattcatgtc gaccacaagc agaacctgta tgccttgacc 2520
cacgagggag aggtgtttca tcagccgcgt gaagcctggc agaatggtgc cgaaagcagc 2580
agctggcaca aactggcgtt gccacagagt gaaagtaagc taaaaagtct ggacatgagc 2640
catgagcaca aaccgattgc cacctttgaa gacggtagcc agcatcagct gaaggctggc 2700
ggctggcacg cctatgcggc acctgaacgc gggccgctgg cggtgggtac cagcggttca 2760
caaaccgtct ttaaccgact aatgcagggg gtgaaaggca aggtgatccc aggcagcggg 2820
ttgacggtta agctctcggc tcagacgggg ggaatgaccg gcgccgaagg gcgcaaggtc 2880
agcagtaaat tttccgaaag gatccgcgcc tatgcgttca acccaacaat gtccacgccg 2940
cgaccgatta aaaatgctgc ttatgccaca cagcacggct ggcaggggcg tgaggggttg 3000
aagccgttgt acgagatgca gggagcgctg attaaacaac tggatgcgca taacgttcgt 3060
cataacgcgc cacagccaga tttgcagagc aaactggaaa ctctggattt aggcgaacat 3120
ggcgcagaat tgcttaacga catgaagcgc ttccgcgacg aactggagca gagtgcaacc 3180
cgttcggtga ccgttttagg tcaacatcag ggagtgctaa aaagcaacgg tgaaatcaat 3240
agcgaattta agccatcgcc cggcaaggcg ttggtccaga gctttaacgt caatcgctct 3300
ggtcaggatc taagcaagtc actgcaacag gcagtacatg ccacgccgcc atccgcagag 3360

agtaaactgc aatccatgct ggggcacttt gtcagtgccg gggtggatat gagtcatcag 3420
aagggcgaga tcccgctggg ccgccagcgc gatccgaatg ataaaaccgc actgaccaaa 3480
tcgcgtttaa ttttagatac cgtgaccatc ggtgaactgc atgaactggc cgataaggcg 3540
aaactggtat ctgaccataa acccgatgcc gatcagataa aacagctgcg ccagcagttc 3600
gatacgctgc gtgaaaagcg gtatgagagc aatccggtga agcattacac cgatatgggc 3660
ttcacccata ataaggcgct ggaagcaaac tatgatgcgg tcaaagcctt tatcaatgcc 3720
tttaagaaag agcaccacgg cgtcaatctg accacgcgta ccgtactgga atcacagggc 3780
agtgcggagc tggcgaagaa gctcaagaat acgctgttgt ccctggacag tggtgaaagt 3840
atgagcttca gccggtcata tggcgggggc gtcagcactg tctttgtgcc tacccttagc 3900
aagaaggtgc cagttccggt gatccccgga gccggcatca cgctggatcg cgcctataac 3960
ctgagcttca gtcgtaccag cggcggattg aacgtcagtt ttggccgcga cggcggggtg 4020
agtggtaaca tcatggtcgc taccggccat gatgtgatgc cctatatgac cggtaagaaa 4080
accagtgcag gtaacgccag tgactggttg agcgcaaaac ataaaatcag cccggacttg 4140
cgtatcggcg ctgctgtgag tggcaccctg caaggaacgc tacaaaacag cctgaagttt 4200
aagctgacag aggatgagct gcctggcttt atccatggct tgacgcatgg cacgttgacc 4260
ccggcagaac tgttgcaaaa ggggatcgaa catcagatga agcagggcag caaactgacg 4320
tttagcgtcg atacctcggc aaatctggat ctgcgtgccg gtatcaatct gaacgaagac 4380
ggcagtaaac caaatggtgt cactgcccgt gtttctgccg ggctaagtgc atcggcaaac 4440
ctggccgccg gctcgcgtga acgcagcacc acctctggcc agtttggcag cacgacttcg 4500
gccagcaata accgcccaac cttcctcaac ggggtcggcg cgggtgctaa cctgacggct 4560
gctttagggg ttgcccattc atctacgcat gaagggaaac cggtcgggat cttcccggca 4620
tttacctcga ccaatgtttc ggcagcgctg gcgctggata accgtacctc acagagtatc 4680
agcctggaat tgaagcgcgc ggagccggtg accagcaacg atatcagcga gttgacctcc 4740
acgctgggaa aacactttaa ggatagcgcc acaacgaaga tgcttgccgc tctcaaagag 4800
ttagatgacg ctaagcccgc tgaacaactg catattttac agcagcattt cagtgcaaaa 4860
gatgtcgtcg gtgatgaacg ctacgaggcg gtgcgcaacc tgaaaaaact ggtgatacgt 4920
caacaggctg cggacagcca cagcatggaa ttaggatctg ccagtcacag cacgacctac 4980
aataatctgt cgagaataaa taatgacggc attgtcgagc tgctacacaa acatttcgat 5040
gcggcattac cagcaagcag tgccaaacgt cttggtgaaa tgatgaataa cgatccggca 5100
ctgaaagata ttattaagca gctgcaaagt acgccgttca gcagcgccag cgtgtcgatg 5160
gagctgaaag atggtctgcg tgagcagacg gaaaaagcaa tactggacgg taaggtcggt 5220
cgtgaagaag tgggagtact tttccaggat cgtaacaact tgcgtgttaa atcggtcagc 5280
gtcagtcagt ccgtcagcaa aagcgaaggc ttcaataccc cagcgctgtt actggggacg 5340
agcaacagcg ctgctatgag catggagcgc aacatcggaa ccattaattt taaatacggc 5400
caggatcaga acaccccacg gcgatttacc ctggagggtg gaatagctca ggctaatccg 5460
caggtcgcat ctgcgcttac tgatttgaag aaggaagggc tggaaatgaa gagctaa 5517

<210> 8
<211> 1838
<212> PRT
<213> Erwinia amylovora

<400> 8
Met Glu Leu Lys Ser Leu Gly Thr Glu His Lys Ala Ala Val His Thr
1 5 10 15

Ala Ala His Asn Pro Val Gly His Gly Val Ala Leu Gln Gln Gly Ser
20 25 30

Ser Ser Ser Ser Pro Gln Asn Ala Ala Ala Ser Leu Ala Ala Glu Gly
35 40 45

Lys Asn Arg Gly Lys Met Pro Arg Ile His Gln Pro Ser Thr Ala Ala
50 55 60

Asp Gly Ile Ser Ala Ala His Gln Gln Lys Lys Ser Phe Ser Leu Arg
65 70 75 80

Gly Cys Leu Gly Thr Lys Lys Phe Ser Arg Ser Ala Pro Gln Gly Gln
85 90 95

Pro Gly Thr Thr His Ser Lys Gly Ala Thr Leu Arg Asp Leu Leu Ala
100 105 110

Arg Asp Asp Gly Glu Thr Gln His Glu Ala Ala Ala Pro Asp Ala Ala
115 120 125

Arg Leu Thr Arg Ser Gly Gly Val Lys Arg Arg Asn Met Asp Asp Met
130 135 140

Ala Gly Arg Pro Met Val Lys Gly Gly Ser Gly Glu Asp Lys Val Pro
145 150 155 160

Thr Gln Gln Lys Arg His Gln Leu Asn Asn Phe Gly Gln Met Arg Gln
165 170 175

Thr Met Leu Ser Lys Met Ala His Pro Ala Ser Ala Asn Ala Gly Asp
180 185 190

Arg Leu Gln His Ser Pro Pro His Ile Pro Gly Ser His His Glu Ile
195 200 205

Lys Glu Glu Pro Val Gly Ser Thr Ser Lys Ala Thr Thr Ala His Ala
210 215 220

Asp Arg Val Glu Ile Ala Gln Glu Asp Asp Asp Ser Glu Phe Gln Gln
225 230 235 240

Leu His Gln Gln Arg Leu Ala Arg Glu Arg Glu Asn Pro Pro Gln Pro
245 250 255

Pro Lys Leu Gly Val Ala Thr Pro Ile Ser Ala Arg Phe Gln Pro Lys
260 265 270

Leu Thr Ala Val Ala Glu Ser Val Leu Glu Gly Thr Asp Thr Thr Gln
275 280 285

Ser Pro Leu Lys Pro Gln Ser Met Leu Lys Gly Ser Gly Ala Gly Val
290 295 300

Thr Pro Leu Ala Val Thr Leu Asp Lys Gly Lys Leu Gln Leu Ala Pro
305 310 315 320

Asp Asn Pro Pro Ala Leu Asn Thr Leu Leu Lys Gln Thr Leu Gly Lys
325 330 335

Asp Thr Gln His Tyr Leu Ala His His Ala Ser Ser Asp Gly Ser Gln
340 345 350

His Leu Leu Leu Asp Asn Lys Gly His Leu Phe Asp Ile Lys Ser Thr
355 360 365

Ala Thr Ser Tyr Ser Val Leu His Asn Ser His Pro Gly Glu Ile Lys
370 375 380

Gly Lys Leu Ala Gln Ala Gly Thr Gly Ser Val Ser Val Asp Gly Lys
385 390 395 400

Ser Gly Lys Ile Ser Leu Gly Ser Gly Thr Gln Ser His Asn Lys Thr
405 410 415

Met Leu Ser Gln Pro Gly Glu Ala His Arg Ser Leu Leu Thr Gly Ile
420 425 430

Trp Gln His Pro Ala Gly Ala Ala Arg Pro Gln Gly Glu Ser Ile Arg
435 440 445

Leu His Asp Asp Lys Ile His Ile Leu His Pro Glu Leu Gly Val Trp
450 455 460

Gln Ser Ala Asp Lys Asp Thr His Ser Gln Leu Ser Arg Gln Ala Asp
465 470 475 480

Gly Lys Leu Tyr Ala Leu Lys Asp Asn Arg Thr Leu Gln Asn Leu Ser
485 490 495

Asp Asn Lys Ser Ser Glu Lys Leu Val Asp Lys Ile Lys Ser Tyr Ser
500 505 510

Val Asp Gln Arg Gly Gln Val Ala Ile Leu Thr Asp Thr Pro Gly Arg
515 520 525

His Lys Met Ser Ile Met Pro Ser Leu Asp Ala Ser Pro Glu Ser His
530 535 540

Ile Ser Leu Ser Leu His Phe Ala Asp Ala His Gln Gly Leu Leu His
545 550 555 560

Gly Lys Ser Glu Leu Glu Ala Gln Ser Val Ala Ile Ser His Gly Arg
565 570 575

Leu Val Val Ala Asp Ser Glu Gly Lys Leu Phe Ser Ala Ala Ile Pro
580 585 590

Lys Gln Gly Asp Gly Asn Glu Leu Lys Met Lys Ala Met Pro Gln His
595 600 605

Ala Leu Asp Glu His Phe Gly His Asp His Gln Ile Ser Gly Phe Phe
610 615 620

His Asp Asp His Gly Gln Leu Asn Ala Leu Val Lys Asn Asn Phe Arg
625 630 635 640

Gln Gln His Ala Cys Pro Leu Gly Asn Asp His Gln Phe His Pro Gly
645 650 655

Trp Asn Leu Thr Asp Ala Leu Val Ile Asp Asn Gln Leu Gly Leu His
660 665 670

His Thr Asn Pro Glu Pro His Glu Ile Leu Asp Met Gly His Leu Gly
675 680 685

Ser Leu Ala Leu Gln Glu Gly Lys Leu His Tyr Phe Asp Gln Leu Thr
690 695 700

Lys Gly Trp Thr Gly Ala Glu Ser Asp Cys Lys Gln Leu Lys Lys Gly
705 710 715 720

Leu Asp Gly Ala Ala Tyr Leu Leu Lys Asp Gly Glu Val Lys Arg Leu
725 730 735

Asn Ile Asn Gln Ser Thr Ser Ser Ile Lys His Gly Thr Glu Asn Val
740 745 750

Phe Ser Leu Pro His Val Arg Asn Lys Pro Glu Pro Gly Asp Ala Leu
755 760 765

Gln Gly Leu Asn Lys Asp Asp Lys Ala Gln Ala Met Ala Val Ile Gly
770 775 780

Val Asn Lys Tyr Leu Ala Leu Thr Glu Lys Gly Asp Ile Arg Ser Phe
785 790 795 800

Gln Ile Lys Pro Gly Thr Gln Gln Leu Glu Arg Pro Ala Gln Thr Leu
805 810 815

Ser Arg Glu Gly Ile Ser Gly Glu Leu Lys Asp Ile His Val Asp His
820 825 830

Lys Gln Asn Leu Tyr Ala Leu Thr His Glu Gly Glu Val Phe His Gln
835 840 845

Pro Arg Glu Ala Trp Gln Asn Gly Ala Glu Ser Ser Ser Trp His Lys
850 855 860

Leu Ala Leu Pro Gln Ser Glu Ser Lys Leu Lys Ser Leu Asp Met Ser
865 870 875 880

His Glu His Lys Pro Ile Ala Thr Phe Glu Asp Gly Ser Gln His Gln
885 890 895

Leu Lys Ala Gly Gly Trp His Ala Tyr Ala Ala Pro Glu Arg Gly Pro
900 905 910

Leu Ala Val Gly Thr Ser Gly Ser Gln Thr Val Phe Asn Arg Leu Met
915 920 925

Gln Gly Val Lys Gly Lys Val Ile Pro Gly Ser Gly Leu Thr Val Lys
930 935 940

Leu Ser Ala Gln Thr Gly Gly Met Thr Gly Ala Glu Gly Arg Lys Val
945 950 955 960

Ser Ser Lys Phe Ser Glu Arg Ile Arg Ala Tyr Ala Phe Asn Pro Thr
965 970 975

Met Ser Thr Pro Arg Pro Ile Lys Asn Ala Ala Tyr Ala Thr Gln His
980 985 990

Gly Trp Gln Gly Arg Glu Gly Leu Lys Pro Leu Tyr Glu Met Gln Gly
995 1000 1005

Ala Leu Ile Lys Gln Leu Asp Ala His Asn Val Arg His Asn Ala Pro
1010 1015 1020

Gln Pro Asp Leu Gln Ser Lys Leu Glu Thr Leu Asp Leu Gly Glu His
1025 1030 1035 1040

Gly Ala Glu Leu Leu Asn Asp Met Lys Arg Phe Arg Asp Glu Leu Glu
1045 1050 1055

Gln Ser Ala Thr Arg Ser Val Thr Val Leu Gly Gln His Gln Gly Val
1060 1065 1070

Leu Lys Ser Asn Gly Glu Ile Asn Ser Glu Phe Lys Pro Ser Pro Gly
1075 1080 1085

Lys Ala Leu Val Gln Ser Phe Asn Val Asn Arg Ser Gly Gln Asp Leu
1090 1095 1100

Ser Lys Ser Leu Gln Gln Ala Val His Ala Thr Pro Pro Ser Ala Glu
1105 1110 1115 1120

Ser Lys Leu Gln Ser Met Leu Gly His Phe Val Ser Ala Gly Val Asp
1125 1130 1135

Met Ser His Gln Lys Gly Glu Ile Pro Leu Gly Arg Gln Arg Asp Pro
1140 1145 1150

Asn Asp Lys Thr Ala Leu Thr Lys Ser Arg Leu Ile Leu Asp Thr Val
1155 1160 1165

Thr Ile Gly Glu Leu His Glu Leu Ala Asp Lys Ala Lys Leu Val Ser
1170 1175 1180

Asp His Lys Pro Asp Ala Asp Gln Ile Lys Gln Leu Arg Gln Gln Phe
1185 1190 1195 1200

Asp Thr Leu Arg Glu Lys Arg Tyr Glu Ser Asn Pro Val Lys His Tyr
1205 1210 1215

Thr Asp Met Gly Phe Thr His Asn Lys Ala Leu Glu Ala Asn Tyr Asp
1220 1225 1230

Ala Val Lys Ala Phe Ile Asn Ala Phe Lys Lys Glu His His Gly Val
1235 1240 1245

Asn Leu Thr Thr Arg Thr Val Leu Glu Ser Gln Gly Ser Ala Glu Leu
1250 1255 1260

Ala Lys Lys Leu Lys Asn Thr Leu Leu Ser Leu Asp Ser Gly Glu Ser
1265 1270 1275 1280

Met Ser Phe Ser Arg Ser Tyr Gly Gly Gly Val Ser Thr Val Phe Val
1285 1290 1295

Pro Thr Leu Ser Lys Lys Val Pro Val Pro Val Ile Pro Gly Ala Gly
1300 1305 1310

Ile Thr Leu Asp Arg Ala Tyr Asn Leu Ser Phe Ser Arg Thr Ser Gly
1315 1320 1325

Gly Leu Asn Val Ser Phe Gly Arg Asp Gly Gly Val Ser Gly Asn Ile
1330 1335 1340

Met Val Ala Thr Gly His Asp Val Met Pro Tyr Met Thr Gly Lys Lys
1345 1350 1355 1360

Thr Ser Ala Gly Asn Ala Ser Asp Trp Leu Ser Ala Lys His Lys Ile
1365 1370 1375

Ser Pro Asp Leu Arg Ile Gly Ala Ala Val Ser Gly Thr Leu Gln Gly
1380 1385 1390

Thr Leu Gln Asn Ser Leu Lys Phe Lys Leu Thr Glu Asp Glu Leu Pro
1395 1400 1405

Gly Phe Ile His Gly Leu Thr His Gly Thr Leu Thr Pro Ala Glu Leu
1410 1415 1420

Leu Gln Lys Gly Ile Glu His Gln Met Lys Gln Gly Ser Lys Leu Thr
1425 1430 1435 1440

Phe Ser Val Asp Thr Ser Ala Asn Leu Asp Leu Arg Ala Gly Ile Asn
1445 1450 1455

Leu Asn Glu Asp Gly Ser Lys Pro Asn Gly Val Thr Ala Arg Val Ser
1460 1465 1470

Ala Gly Leu Ser Ala Ser Ala Asn Leu Ala Ala Gly Ser Arg Glu Arg
1475 1480 1485

Ser Thr Thr Ser Gly Gln Phe Gly Ser Thr Thr Ser Ala Ser Asn Asn
1490 1495 1500

Arg Pro Thr Phe Leu Asn Gly Val Gly Ala Gly Ala Asn Leu Thr Ala
1505 1510 1515 1520

Ala Leu Gly Val Ala His Ser Ser Thr His Glu Gly Lys Pro Val Gly
1525 1530 1535

Ile Phe Pro Ala Phe Thr Ser Thr Asn Val Ser Ala Ala Leu Ala Leu
1540 1545 1550

Asp Asn Arg Thr Ser Gln Ser Ile Ser Leu Glu Leu Lys Arg Ala Glu
1555 1560 1565

Pro Val Thr Ser Asn Asp Ile Ser Glu Leu Thr Ser Thr Leu Gly Lys
1570 1575 1580

His Phe Lys Asp Ser Ala Thr Thr Lys Met Leu Ala Ala Leu Lys Glu
1585 1590 1595 1600

Leu Asp Asp Ala Lys Pro Ala Glu Gln Leu His Ile Leu Gln Gln His
1605 1610 1615

Phe Ser Ala Lys Asp Val Val Gly Asp Glu Arg Tyr Glu Ala Val Arg
1620 1625 1630

Asn Leu Lys Lys Leu Val Ile Arg Gln Gln Ala Ala Asp Ser His Ser
1635 1640 1645

Met Glu Leu Gly Ser Ala Ser His Ser Thr Thr Tyr Asn Asn Leu Ser
1650 1655 1660

Arg Ile Asn Asn Asp Gly Ile Val Glu Leu Leu His Lys His Phe Asp
1665 1670 1675 1680

Ala Ala Leu Pro Ala Ser Ser Ala Lys Arg Leu Gly Glu Met Met Asn
1685 1690 1695

Asn Asp Pro Ala Leu Lys Asp Ile Ile Lys Gln Leu Gln Ser Thr Pro
1700 1705 1710

Phe Ser Ser Ala Ser Val Ser Met Glu Leu Lys Asp Gly Leu Arg Glu
1715 1720 1725

Gln Thr Glu Lys Ala Ile Leu Asp Gly Lys Val Gly Arg Glu Glu Val
1730 1735 1740

Gly Val Leu Phe Gln Asp Arg Asn Asn Leu Arg Val Lys Ser Val Ser
1745 1750 1755 1760

Val Ser Gln Ser Val Ser Lys Ser Glu Gly Phe Asn Thr Pro Ala Leu
1765 1770 1775

Leu Leu Gly Thr Ser Asn Ser Ala Ala Met Ser Met Glu Arg Asn Ile
1780 1785 1790

Gly Thr Ile Asn Phe Lys Tyr Gly Gln Asp Gln Asn Thr Pro Arg Arg
1795 1800 1805

Phe Thr Leu Glu Gly Gly Ile Ala Gln Ala Asn Pro Gln Val Ala Ser
1810 1815 1820

Ala Leu Thr Asp Leu Lys Lys Glu Gly Leu Glu Met Lys Ser
1825 1830 1835

<210> 9
<211> 420
<212> DNA
<213> Erwinia amylovora

<400> 9
atgacatcgt cacagcagcg ggttgaaagg tttttacagt atttctccgc cgggtgtaaa 60
acgcccatac atctgaaaga cggggtgtgc gccctgtata acgaacaaga tgaggaggcg 120
gcggtgctgg aagtaccgca acacagcgac agcctgttac tacactgccg aatcattgag 180
gctgacccac aaacttcaat aaccctgtat tcgatgctat tacagctgaa ttttgaaatg 240
gcggccatgc gcggctgttg gctggcgctg gatgaactgc acaacgtgcg tttatgtttt 300
cagcagtcgc tggagcatct ggatgaagca agttttagcg atatcgttag cggcttcatc 360
gaacatgcgg cagaagtgcg tgagtatata gcgcaattag acgagagtag cgcggcataa 420

<210> 10
<211> 139
<212> PRT
<213> Erwinia amylovora

<400> 10
Met Thr Ser Ser Gln Gln Arg Val Glu Arg Phe Leu Gln Tyr Phe Ser
1 5 10 15

Ala Gly Cys Lys Thr Pro Ile His Leu Lys Asp Gly Val Cys Ala Leu
20 25 30

Tyr Asn Glu Gln Asp Glu Glu Ala Ala Val Leu Glu Val Pro Gln His
35 40 45

Ser Asp Ser Leu Leu Leu His Cys Arg Ile Ile Glu Ala Asp Pro Gln
50 55 60

Thr Ser Ile Thr Leu Tyr Ser Met Leu Leu Gln Leu Asn Phe Glu Met
65 70 75 80

Ala Ala Met Arg Gly Cys Trp Leu Ala Leu Asp Glu Leu His Asn Val
85 90 95

Arg Leu Cys Phe Gln Gln Ser Leu Glu His Leu Asp Glu Ala Ser Phe
100 105 110

Ser Asp Ile Val Ser Gly Phe Ile Glu His Ala Ala Glu Val Arg Glu
115 120 125

Tyr Ile Ala Gln Leu Asp Glu Ser Ser Ala Ala
130 135

<210> 11
<211> 341
<212> PRT
<213> Pseudomonas syringae

<400> 11
Met Gln Ser Leu Ser Leu Asn Ser Ser Ser Leu Gln Thr Pro Ala Met
1 5 10 15

Ala Leu Val Leu Val Arg Pro Glu Ala Glu Thr Thr Gly Ser Thr Ser
20 25 30

Ser Lys Ala Leu Gln Glu Val Val Val Lys Leu Ala Glu Glu Leu Met
35 40 45

Arg Asn Gly Gln Leu Asp Asp Ser Ser Pro Leu Gly Lys Leu Leu Ala
50 55 60

Lys Ser Met Ala Ala Asp Gly Lys Ala Gly Gly Gly Ile Glu Asp Val
65 70 75 80

Ile Ala Ala Leu Asp Lys Leu Ile His Glu Lys Leu Gly Asp Asn Phe
85 90 95

Gly Ala Ser Ala Asp Ser Ala Ser Gly Thr Gly Gln Gln Asp Leu Met
100 105 110

Thr Gln Val Leu Asn Gly Leu Ala Lys Ser Met Leu Asp Asp Leu Leu
115 120 125

Thr Lys Gln Asp Gly Gly Thr Ser Phe Ser Glu Asp Asp Met Pro Met
130 135 140

Leu Asn Lys Ile Ala Gln Phe Met Asp Asp Asn Pro Ala Gln Phe Pro
145 150 155 160

Lys Pro Asp Ser Gly Ser Trp Val Asn Glu Leu Lys Glu Asp Asn Phe
165 170 175

Leu Asp Gly Asp Glu Thr Ala Ala Phe Arg Ser Ala Leu Asp Ile Ile
180 185 190

Gly Gln Gln Leu Gly Asn Gln Gln Ser Asp Ala Gly Ser Leu Ala Gly

195 200 205

Thr Gly Gly Gly Leu Gly Thr Pro Ser Ser Phe Ser Asn Asn Ser Ser
210 215 220

Val Met Gly Asp Pro Leu Ile Asp Ala Asn Thr Gly Pro Gly Asp Ser
225 230 235 240

Gly Asn Thr Arg Gly Glu Ala Gly Gln Leu Ile Gly Glu Leu Ile Asp
245 250 255

Arg Gly Leu Gln Ser Val Leu Ala Gly Gly Gly Leu Gly Thr Pro Val
260 265 270

Asn Thr Pro Gln Thr Gly Thr Ser Ala Asn Gly Gly Gln Ser Ala Gln
275 280 285

Asp Leu Asp Gln Leu Leu Gly Gly Leu Leu Leu Lys Gly Leu Glu Ala
290 295 300

Thr Leu Lys Asp Ala Gly Gln Thr Gly Thr Asp Val Gln Ser Ser Ala
305 310 315 320

Ala Gln Ile Ala Thr Leu Leu Val Ser Thr Leu Leu Gln Gly Thr Arg
325 330 335

Asn Gln Ala Ala Ala
340

<210> 12
<211> 1026
<212> DNA
<213> Pseudomonas syringae

<400> 12
atgcagagtc tcagtcttaa cagcagctcg ctgcaaaccc cggcaatggc ccttgtcctg 60
gtacgtcctg aagccgagac gactggcagt acgtcgagca aggcgcttca ggaagttgtc 120
gtgaagctgg ccgaggaact gatgcgcaat ggtcaactcg acgacagctc gccattggga 180
aaactgttgg ccaagtcgat ggccgcagat ggcaaggcgg gcggcggtat tgaggatgtc 240
atcgctgcgc tggacaagct gatccatgaa aagctcggtg acaacttcgg cgcgtctgcg 300
gacagcgcct cgggtaccgg acagcaggac ctgatgactc aggtgctcaa tggcctggcc 360
aagtcgatgc tcgatgatct tctgaccaag caggatggcg ggacaagctt ctccgaagac 420
gatatgccga tgctgaacaa gatcgcgcag ttcatggatg acaatcccgc acagtttccc 480
aagccggact cgggctcctg ggtgaacgaa ctcaaggaag acaacttcct tgatggcgac 540
gaaacggctg cgttccgttc ggcactcgac atcattggcc agcaactggg taatcagcag 600
agtgacgctg gcagtctggc agggacgggt ggaggtctgg gcactccgag cagtttttcc 660
aacaactcgt ccgtgatggg tgatccgctg atcgacgcca ataccggtcc cggtgacagc 720

ggcaataccc gtggtgaagc ggggcaactg atcggcgagc ttatcgaccg tggcctgcaa 780
tcggtattgg ccggtggtgg actgggcaca cccgtaaaca ccccgcagac cggtacgtcg 840
gcgaatggcg gacagtccgc tcaggatctt gatcagttgc tgggcggctt gctgctcaag 900
ggcctggagg caacgctcaa ggatgccggg caaacaggca ccgacgtgca gtcgagcgct 960
gcgcaaatcg ccaccttgct ggtcagtacg ctgctgcaag gcacccgcaa tcaggctgca 1020
gcctga 1026

<210> 13
<211> 1729
<212> DNA
<213> Pseudomonas syringae

<400> 13
tccacttcgc tgattttgaa attggcagat tcatagaaac gttcaggtgt ggaaatcagg 60
ctgagtgcgc agatttcgtt gataagggtg tggtactggt cattgttggt catttcaagg 120
cctctgagtg cggtgcggag caataccagt cttcctgctg gcgtgtgcac actgagtcgc 180
aggcataggc atttcagttc cttgcgttgg ttgggcatat aaaaaaagga actttaaaa 240
acagtgcaat gagatgccgg caaaacggga accggtcgct gcgctttgcc actcacttcg 300
agcaagctca accccaaaca tccacatccc tatcgaacgg acagcgatac ggccacttgc 360
tctggtaaac cctggagctg gcgtcggtcc aattgcccac ttagcgaggt aacgcagcat 420
gagcatcggc atcacacccc ggccgcaaca gaccaccacg ccactcgatt tttcggcgct 480
aagcggcaag agtcctcaac caaacacgtt cggcgagcag aacactcagc aagcgatcga 540
cccgagtgca ctgttgttcg gcagcgacac acagaaagac gtcaacttcg gcacgcccga 600
cagcaccgtc cagaatccgc aggacgccag caagcccaac gacagccagt ccaacatcgc 660
taaattgatc agtgcattga tcatgtcgtt gctgcagatg ctcaccaact ccaataaaaa 720
gcaggacacc aatcaggaac agcctgatag ccaggctcct ttccagaaca acggcgggct 780
cggtacaccg tcggccgata gcgggggcgg cggtacaccg gatgcgacag gtggcggcgg 840
cggtgatacg ccaagcgcaa caggcggtgg cggcggtgat actccgaccg caacaggcgg 900
tggcggcagc ggtggcggcg gcacacccac tgcaacaggt ggcggcagcg gtggcacacc 960
cactgcaaca ggcggtggcg agggtggcgt aacaccgcaa atcactccgc agttggccaa 1020
ccctaaccgt acctcaggta ctggctcggt gtcggacacc gcaggttcta ccgagcaagc 1080
cggcaagatc aatgtggtga aagacaccat caaggtcggc gctggcgaag tctttgacgg 1140
ccacggcgca accttcactg ccgacaaatc tatgggtaac ggagaccagg gcgaaaatca 1200
gaagcccatg ttcgagctgg ctgaaggcgc tacgttgaag aatgtgaacc tgggtgagaa 1260
cgaggtcgat ggcatccacg tgaaagccaa aaacgctcag gaagtcacca ttgacaacgt 1320
gcatgcccag aacgtcggtg aagacctgat tacggtcaaa ggcgagggag gcgcagcggt 1380
cactaatctg aacatcaaga acagcagtgc caaaggtgca gacgacaagg ttgtccagct 1440
caacgccaac actcacttga aaatcgacaa cttcaaggcc gacgatttcg gcacgatggt 1500
tcgcaccaac ggtggcaagc agtttgatga catgagcatc gagctgaacg gcatcgaagc 1560
taaccacggc aagttcgccc tggtgaaaag cgacagtgac gatctgaagc tggcaacggg 1620
caacatcgcc atgaccgacg tcaaacacgc ctacgataaa acccaggcat cgacccaaca 1680
caccgagctt tgaatccaga caagtagctt gaaaaaaggg ggtggactc 1729

<210> 14
<211> 424
<212> PRT
<213> Pseudomonas syringae

<400> 14

Met Ser Ile Gly Ile Thr Pro Arg Pro Gln Gln Thr Thr Thr Pro Leu
1   5   10   15

Asp Phe Ser Ala Leu Ser Gly Lys Ser Pro Gln Pro Asn Thr Phe Gly
            20   25   30

Glu Gln Asn Thr Gln Gln Ala Ile Asp Pro Ser Ala Leu Leu Phe Gly
        35   40   45

Ser Asp Thr Gln Lys Asp Val Asn Phe Gly Thr Pro Asp Ser Thr Val
    50   55   60

Gln Asn Pro Gln Asp Ala Ser Lys Pro Asn Asp Ser Gln Ser Asn Ile
65   70   75   80

Ala Lys Leu Ile Ser Ala Leu Ile Met Ser Leu Leu Gln Met Leu Thr
                85   90   95

Asn Ser Asn Lys Lys Gln Asp Thr Asn Gln Glu Gln Pro Asp Ser Gln
            100   105   110

Ala Pro Phe Gln Asn Asn Gly Gly Leu Gly Thr Pro Ser Ala Asp Ser
        115   120   125

Gly Gly Gly Gly Thr Pro Asp Ala Thr Gly Gly Gly Gly Gly Asp Thr
    130   135   140

Pro Ser Ala Thr Gly Gly Gly Gly Gly Asp Thr Pro Thr Ala Thr Gly
145   150   155   160

Gly Gly Gly Ser Gly Gly Gly Gly Thr Pro Thr Ala Thr Gly Gly Gly
                165   170   175

Ser Gly Gly Thr Pro Thr Ala Thr Gly Gly Gly Glu Gly Gly Val Thr
            180   185   190

Pro Gln Ile Thr Pro Gln Leu Ala Asn Pro Asn Arg Thr Ser Gly Thr
        195   200   205

Gly Ser Val Ser Asp Thr Ala Gly Ser Thr Glu Gln Ala Gly Lys Ile
    210   215   220

Asn Val Val Lys Asp Thr Ile Lys Val Gly Ala Gly Glu Val Phe Asp
225   230   235   240

Gly His Gly Ala Thr Phe Thr Ala Asp Lys Ser Met Gly Asn Gly Asp
                245   250   255

Gln Gly Glu Asn Gln Lys Pro Met Phe Glu Leu Ala Glu Gly Ala Thr
260 265 270

Leu Lys Asn Val Asn Leu Gly Glu Asn Glu Val Asp Gly Ile His Val
275 280 285

Lys Ala Lys Asn Ala Gln Glu Val Thr Ile Asp Asn Val His Ala Gln
290 295 300

Asn Val Gly Glu Asp Leu Ile Thr Val Lys Gly Glu Gly Gly Ala Ala
305 310 315 320

Val Thr Asn Leu Asn Ile Lys Asn Ser Ser Ala Lys Gly Ala Asp Asp
325 330 335

Lys Val Val Gln Leu Asn Ala Asn Thr His Leu Lys Ile Asp Asn Phe
340 345 350

Lys Ala Asp Asp Phe Gly Thr Met Val Arg Thr Asn Gly Gly Lys Gln
355 360 365

Phe Asp Asp Met Ser Ile Glu Leu Asn Gly Ile Glu Ala Asn His Gly
370 375 380

Lys Phe Ala Leu Val Lys Ser Asp Ser Asp Asp Leu Lys Leu Ala Thr
385 390 395 400

Gly Asn Ile Ala Met Thr Asp Val Lys His Ala Tyr Asp Lys Thr Gln
405 410 415

Ala Ser Thr Gln His Thr Glu Leu
420

<210> 15
<211> 344
<212> PRT
<213> Pseudomonas solanacearum

<400> 15
Met Ser Val Gly Asn Ile Gln Ser Pro Ser Asn Leu Pro Gly Leu Gln
1 5 10 15

Asn Leu Asn Leu Asn Thr Asn Thr Asn Ser Gln Gln Ser Gly Gln Ser
20 25 30

Val Gln Asp Leu Ile Lys Gln Val Glu Lys Asp Ile Leu Asn Ile Ile

35 40 45

Ala Ala Leu Val Gln Lys Ala Ala Gln Ser Ala Gly Gly Asn Thr Gly
50 55 60

Asn Thr Gly Asn Ala Pro Ala Lys Asp Gly Asn Ala Asn Ala Gly Ala
65 70 75 80

Asn Asp Pro Ser Lys Asn Asp Pro Ser Lys Ser Gln Ala Pro Gln Ser
85 90 95

Ala Asn Lys Thr Gly Asn Val Asp Asp Ala Asn Asn Gln Asp Pro Met
100 105 110

Gln Ala Leu Met Gln Leu Leu Glu Asp Leu Val Lys Leu Leu Lys Ala
115 120 125

Ala Leu His Met Gln Gln Pro Gly Gly Asn Asp Lys Gly Asn Gly Val
130 135 140

Gly Gly Ala Asn Gly Ala Lys Gly Ala Gly Gly Gln Gly Gly Leu Ala
145 150 155 160

Glu Ala Leu Gln Glu Ile Glu Gln Ile Leu Ala Gln Leu Gly Gly Gly
165 170 175

Gly Ala Gly Ala Gly Gly Ala Gly Gly Gly Val Gly Gly Ala Gly Gly
180 185 190

Ala Asp Gly Gly Ser Gly Ala Gly Gly Ala Gly Gly Ala Asn Gly Ala
195 200 205

Asp Gly Gly Asn Gly Val Asn Gly Asn Gln Ala Asn Gly Pro Gln Asn
210 215 220

Ala Gly Asp Val Asn Gly Ala Asn Gly Ala Asp Asp Gly Ser Glu Asp
225 230 235 240

Gln Gly Gly Leu Thr Gly Val Leu Gln Lys Leu Met Lys Ile Leu Asn
245 250 255

Ala Leu Val Gln Met Met Gln Gln Gly Gly Leu Gly Gly Gly Asn Gln
260 265 270

Ala Gln Gly Gly Ser Lys Gly Ala Gly Asn Ala Ser Pro Ala Ser Gly
275 280 285

Ala Asn Pro Gly Ala Asn Gln Pro Gly Ser Ala Asp Asp Gln Ser Ser

290 295 300

Gly Gln Asn Asn Leu Gln Ser Gln Ile Met Asp Val Val Lys Glu Val
305 310 315 320

Val Gln Ile Leu Gln Gln Met Leu Ala Ala Gln Asn Gly Gly Ser Gln
325 330 335

Gln Ser Thr Ser Thr Gln Pro Met
340

<210> 16
<211> 1035
<212> DNA
<213> Pseudomonas solanacearum

<400> 16
atgtcagtcg gaaacatcca gagcccgtcg aacctcccgg gtctgcagaa cctgaacctc 60
aacaccaaca ccaacagcca gcaatcgggc cagtccgtgc aagacctgat caagcaggtc 120
gagaaggaca tcctcaacat catcgcagcc ctcgtgcaga aggccgcaca gtcggcgggc 180
ggcaacaccg gtaacaccgg caacgcgccg gcgaaggacg gcaatgccaa cgcgggcgcc 240
aacgacccga gcaagaacga cccgagcaag agccaggctc cgcagtcggc caacaagacc 300
ggcaacgtcg acgacgccaa caaccaggat ccgatgcaag cgctgatgca gctgctggaa 360
gacctggtga agctgctgaa ggcggccctg cacatgcagc agcccggcgg caatgacaag 420
ggcaacggcg tgggcggtgc caacggcgcc aagggtgccg gcggccaggg cggcctggcc 480
gaagcgctgc aggagatcga gcagatcctc gcccagctcg gcggcggcgg tgctggcgcc 540
ggcggcgcgg gtggcggtgt cggcggtgct ggtggcgcgg atggcggctc cggtgcgggt 600
ggcgcaggcg gtgcgaacgg cgccgacggc ggcaatggcg tgaacggcaa ccaggcgaac 660
ggcccgcaga acgcaggcga tgtcaacggt gccaacggcg cggatgacgg cagcgaagac 720
cagggcggcc tcaccggcgt gctgcaaaag ctgatgaaga tcctgaacgc gctggtgcag 780
atgatgcagc aaggcggcct cggcggcggc aaccaggcgc agggcggctc gaagggtgcc 840
ggcaacgcct cgccggcttc cggcgcgaac ccgggcgcga accagcccgg ttcggcggat 900
gatcaatcgt ccggccagaa caatctgcaa tcccagatca tggatgtggt gaaggaggtc 960
gtccagatcc tgcagcagat gctggcggcg cagaacggcg gcagccagca gtccacctcg 1020
acgcagccga tgtaa 1035

<210> 17
<211> 26
<212> PRT
<213> Xanthomonas campestris pv. glycines

<400> 17
Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Ala Ile Leu Ala
1 5 10 15

Ala Ile Ala Leu Pro Ala Tyr Gln Asp Tyr
20 25

<210> 18
<211> 20
<212> PRT
<213> Xanthomonas campestris pv. pelargonii

<400> 18
```
Ser Ser Gln Gln Ser Pro Ser Ala Gly Ser Glu Gln Gln Leu Asp Gln
 1               5                  10                  15

Leu Leu Ala Met
            20
```